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# THE SYNERGISM OF ANESTHETICS AND HYPNOTICS WITH CURARE AND CURARE-LIKE ALKALOIDS

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Curare and curare-like alkaloids have found increasing use in neurology to relieve or abolish the spasticity of muscles in various brain and spinal cord diseases (1) and particularly in surgery to improve muscle relaxation during inhalation anesthesia as well as during narcosis induced by barbiturates. Clinical observations on patients under ether, avertin or pentothal anesthesia and also on those subjected to nitrous oxide, ethylene or cyclopropane have revealed that combinations of these substances with curare produce excellent surgical relaxation (2, 3). Gray and Halton (4) have demonstrated in patients a definite synergistic action between tubocurarine and the barbiturates. However, in none of these reports has there been an attempt at a pharmacological analysis to explain the reason for this effect.

Feitelberg and Pick (5), Pick and Unna (6) and recently McIntyre, Dunn and Tullar (7) have demonstrated that curare and certain curare-like compounds produce definite changes in the electrical brain potential. The investigation reported in this communication was undertaken to determine whether the enhanced muscular relaxation resulting from the combined action of curare with hypnotics or anesthetics should be attributed to a central or peripheral action of the drugs.

**METHOD.** The investigations were carried out on mice, rabbits and cats under ether, urethane, chloralhydrate, phenobarbital sodium, morphine and pentobarbital sodium anesthesia. In certain experiments scopolamine and a combination of morphine and scopolamine were used. The curare and curare-like alkaloids used were strychnos curare (Merek), crystallized d-tubocurarine chloride,<sup>1</sup> Intocostrin (Squibb), crystallized dihydro- $\beta$ -erythroidine hydrobromide (Merek), quinine methochloride, quinine ethochloride<sup>1</sup> and quinine sulfate. In most cases the animals were first injected subcutaneously or intraperitoneally with the anesthetic or hypnotic, and after a suitable period of time the alkaloid was administered either subcutaneously or intravenously. In some experiments the order of administration was reversed.

To be sure that only a definite and distinct enhancement of the action of narcotics through combination with alkaloids was being studied, doses of the narcotics, hypnotics, and alkaloids which alone showed no effect on normal animals were employed. The time required to produce loss of righting reflexes and complete relaxation of neck, abdominal and leg muscles was noted. To obtain more complete knowledge of the state of narcosis produced, all changes occurring spontaneously during the period of drug action were noted. Although the method of evaluating the degree of paralysis or relaxation was only an approximate one, it was sufficient since it was our purpose to detect only positive action.

<sup>1</sup>The senior author (E. P. Pick) wishes to express his thanks to Dr. O. Wintersteiner of the Squibb Institute for Medical Research for the d-tubocurarine and to Dr. Oliver Kamm of Parke-Davis and Company for the quinine-ethochloride used in this study.

Hence a most valuable indication of a synergistic action was the paralysis which immediately followed intravenous injection of the alkaloid in animals previously treated with narcotics or anesthetics.

**A. CURARE.** *I. Action of curare with ether in mice.* A suitable sub-effective ether concentration for mice (0.25 cc. of ether in a 1.5 liter bottle) was first determined. Groups of 3 to 10 mice kept in this ether concentration for one-half hour or longer were not anesthetized. Also it was established that the subcutaneous administration of 0.30 mg. per kg. or less of d-tubocurarine chloride, 1.8 mg. per kg. or less of Intocostrin, or 1.8 cc. per kg. of a solution of strychnos curare produced no noticeable effects in mice.

When mice pretreated with these ineffective doses of the different curare preparations were exposed, 10-28 minutes later, to the sub-effective ether concentration a considerably enhanced sensitivity to ether was observed. Within 10 to 15 minutes after exposure all animals exhibited complete motor paralysis without appreciable demonstrable anesthesia; in some cases death occurred.

The state of alteration depends not only on the amounts of curare and ether administered, but also on the length of time which elapses between the injection of alkaloid and exposure to anesthetic. That the concentration of ether is critical was demonstrated in an experiment in which mice dosed with the same amount of strychnos curare (2.0 cc. per kg.) were exposed to different concentrations of ether. When a concentration of 0.1 cc. ether in a 1.5 liter bottle was employed only a slight degree of narcosis was noted. However a concentration of 0.25 cc. ether in the same volume of air produced a deep prolonged narcosis and a death rate of 62.5%. In another series of experiments it was shown that if the period between the injection of curare and exposure to ether was permitted to extend for 50 to 100 minutes, it was impossible to demonstrate increased sensitivity to ether even when the amount of previously administered curare was in itself sufficient to produce a slight degree of paralysis. Apparently within this time, a greater part of the curare was either excreted or destroyed.

*II. Action of d-tubocurarine chloride with phenobarbital sodium, trichlorobutanol and urethane in mice.* Mice prepared by injecting subcutaneously d-tubocurarine chloride (0.3 mg. per kg.) were treated after a 10-15 minute interval with phenobarbital sodium (40-80 mg. per kg.) or trichlorobutanol (80 mg. per kg.). In some experiments the order of treatment was reversed. The doses of alkaloid and hypnotics employed when administered alone, produced no visible effect on the animals.

In the experiments with alkaloid and phenobarbital sodium or alkaloid and trichlorobutanol a narcotizing effect similar to the previously described curare-ether synergism was achieved. It made no difference in the enhancement of the narcotizing action whether pretreatment was made with the alkaloid or with either of the hypnotics providing that the time between the application of the two drugs was limited to 10-15 minutes. However, the narcotizing (paralyzing) action seemed to be more dependent on the administration of a sufficient dose of the hypnotic than on the amounts of curare injected. This was made evident by the poor and delayed narcotizing action obtained in experi-

ments in which 0.4 mg. per kg. and 0.3 mg. per kg. of d-tubocurarine chloride were combined with only 40 mg. per kg. phenobarbital-sodium.

Contrary to the enhancement of effect produced by phenobarbital or trichlorobutanol combined with d-tubocurarine chloride, it was impossible to demonstrate a similar effect with small doses of urethane. Mice treated with urethane (0.2-0.4 mg. per kg.) and later injected with d-tubocurarine chloride (0.2 mg. per kg.) or with dihydro- $\beta$ -erythroidine hydrobromide (2.0 mg. per kg.) showed no increased hypnotic action.

*III. Action of curare with pentobarbital sodium in cats.* These experiments were performed on cats weighing 2.5-3.5 kg. injected intraperitoneally with pentobarbital sodium solution in doses of 5, 10, 15, 20 mg. per kg. Pentobarbital alone in these doses did not produce a deep narcosis or anesthesia. Animals dosed with 10 mg. per kg. of pentobarbital, (approximately one-third of the dose necessary for full anesthesia) walked unsteadily, but showed no signs of sleep or change in position such as lying on their sides. Thirty to sixty minutes after the injection these cats were again normal. This dose of pentobarbital was employed in most of the following experiments.

In the first series of experiments d-tubocurarine chloride in doses of 2.0, 4.0 and 8.0 mg. per kg. was injected *subcutaneously* into cats pretreated with pentobarbital (10 mg. per kg.). No enhancement of the narcotizing action of the hypnotic was obtained. It is probable that the relatively slow absorption following subcutaneous administration combined with rapid excretion or destruction of curare prevented the attainment of a blood level of the alkaloid sufficiently high to establish narcosis when combined with only slightly effective amounts of pentobarbital. Therefore it was considered that more favorable results might be expected following *intravenous* administration of the alkaloids.

Results of experiments in cats prepared with pentobarbital sodium and injected *intravenously* with d-tubocurarine chloride are shown in table 1. Cats pretreated intraperitoneally with 5 mg. per kg. pentobarbital and later injected intravenously with 0.04 mg. per kg. of the alkaloid showed no visible enhancement of narcosis, (experiment 5, table 1). On the other hand, cats prepared by the intraperitoneal administration of 20 mg. per kg. pentobarbital were immediately paralyzed following the intravenous injection of 0.05 mg. per kg. d-tubocurarine chloride (experiment 10, table 1) despite the fact that 20 mg. per kg. pentobarbital alone was not able to produce complete muscular relaxation within 15-20 minutes after injection.

Doses of crystalline d-tubocurarine chloride of 0.05-0.10 mg. per kg. intravenously, representing only 6 to 12 per cent of the dose causing complete paralysis in cats, when combined with small amounts of pentobarbital usually produced an immediate relaxation of all muscles with the exception of the diaphragm. A similar ratio between the relaxing and the completely paralyzing doses was demonstrated in experiments with strychnos curare (Merek). As little as 10 per cent of the fatal intravenous dose for cats (about 2 mg. per kg.) produced complete relaxation immediately after intravenous injection into cats pretreated with pentobarbital. No paralysis of the diaphragm or change in respiration was noted in these animals.

The enhancement of the curare effect is therefore apparently linked with the first stages of curare action on the striated muscles of the extremities, abdominal wall and the neck. It is probable that the amounts of curare used in our experi-

TABLE 1  
*Curare experiments in cats pretreated with pentobarbital sodium*

NO. OF EXPERIMENT	WEIGHT OF CAT IN KG.	PENTOBARBITAL SODIUM  <i>mg./kg. (i.p.)</i>	INTERVAL BETWEEN PENTOBARBITAL SODIUM AND CURARE INJECTION  <i>minutes</i>	d-TU- BOCURARINE Cl  <i>mg./kg. (i.s.)</i>	RESULTS
1	2.7	2.0	25	0.037	20 min. later—asleep. 2 hrs. later—mostly asleep.
2	2.0	2.0	30	0.05	50 min. later—asleep. 2 hrs. later—not asleep.
3	2.5	2.0	15	0.08	Not asleep.
4	2.1	2.0	20	0.10	Not asleep.
5	2.5	5.0	2	0.04	Not affected.
6	3.1	10.0	2	0.1	Immediately sleepy, completely relaxed, not anesthetized. 5 min. after curare injection—0.1 mg. <i>prostigmine i.v.</i> : 2 min. later tried to walk, pupils large, 9 min. later—walked unsteadily. 1 hr. 13 min. later—normal.
7	2.6	10.0	5	0.076	5 min. later—sleepy—on side. 30 min. later—deep sleep. 2 hrs. later—deep sleep, but not anesthetized.
8	2.65	10.0	10	0.075	Immediately lies on side, moved only head. 2 min. later cannot raise head, pupils small. 10 min. later—sleepy. 25 min. later—awakens unable to walk. 40 min. later—walking unsteadily, recovering.
9	2.7	20.0	7	0.1	Paralyzed immediately, on side, unable to move, completely relaxed. 15 min. later—narcosis. 30 min. later—begins to move.

TABLE 1—*Concluded*

NO. OF EXPERIMENT	WEIGHT OF CAT IN KG.	PENTOBARBITAL SODIUM	INTERVAL BETWEEN PENTOBARBITAL SODIUM AND CURARE INJECTION	d-TU-BOCURARINE Cl	RESULTS
		mg./kg. (i.p.)	minutes	mg./kg. (i.v.)	
10	3.0	20.0	4	0.05	2 min. later—on side, not completely relaxed, no anesthesia. 7 min. later—completely paralyzed, relaxed, narcosis. 52 min. later—unchanged. 1 hr. 22 min. later—0.17 mg. <i>prostigmine</i> cat moved legs violently, tried to move head, still affected by <i>nembutal</i> .
11	2.8	—	—	0.1	No effect.
12	2.6	—	—	0.2	After 1 min.—lying on side. After 3 min. fully recovered.
13	2.6	—	—	0.2	No effect.
14	2.6	—	—	0.4	No effect.
15	2.6	—	—	0.8	Cat almost died of respiratory failure. Immediately given 0.25 mg. <i>prostigmine</i> i.v.—after 24 min.—attempted to walk.

ments were too small to affect the function of the more resistant diaphragm and other respiratory muscles. It may also be considered that the increase of the curare effect depends on the central action of the hypnotics despite the fact that curare itself is mainly responsible for the selection of muscles and the disappearance of tonus and motility. In this connection the effect of *prostigmine* on cats paralyzed after treatment with pentobarbital and curare should be mentioned. While an intravenous injection of 0.25 mg. *prostigmine* immediately antagonizes the fatal action of 2 mg. per kg. strychnos curare and produces a complete recovery within 5 minutes the *prostigmine* action on the cats treated with pentobarbital plus curare is limited to the peripheral effects, permitting movements of the extremities and head, although the animals still appear sleepy. The control of the combined hypnotic and curare action is not considerably influenced by *prostigmine*. This is in accordance with experiments on frogs made by Pick and Unna (4) which showed the independence of the central and peripheral actions of d-tubocurarine chloride.

B. ERYTHROIDINE. Extensive pharmacological investigations concerning al-



kaloids of erythrina were performed by Unna and Greslin (8), by Unna, Kniazuk and Greslin (9), and Pick and Unna (6). It was shown that one of the most potent erythrina alkaloids, dihydro- $\beta$ -erythroidine hydrobromide, produced the same central and peripheral effects as d-tubocurarine chloride; and that while its peripheral effects were counteracted by the administration of prostigmine, such treatment did not restore the brain potentials or the synaptic transmission in the brain.

*I. Action of dihydro- $\beta$ -erythroidine hydrobromide with ether in mice.* Mice weighing 20–30 gms. were injected subcutaneously with small doses of dihydro- $\beta$ -erythroidine hydrobromide and 7–20 minutes later were exposed to a low ether concentration (0.25 cc. ether in a 1.5 liter bottle). While amounts of 4, 6, 16, 20, 24, mg. per kg. respectively of dihydro- $\beta$ -erythroidine were found to be ineffective or to produce only a slight temporary paralysis when administered alone, the same amounts combined with ether not only paralyzed, but occasionally killed the mice. Thus ether enhanced the action of dihydro- $\beta$ -erythroidine in a manner similar to that demonstrated in the preceding experiments with curare. On the other hand, the subcutaneous injection of phenobarbital sodium produced only a slight and doubtful enhancement of effect in dihydro- $\beta$ -erythroidine treated mice.

*II. Action of dihydro- $\beta$ -erythroidine hydrobromide with pentobarbital sodium in cats.* Cats premedicated with pentobarbital sodium and 5 to 15 minutes later injected *subcutaneously* with dihydro- $\beta$ -erythroidine hydrobromide even in considerable amounts showed a no greater narcotizing or paralyzing effect than could be demonstrated with the pentobarbital sodium alone. A similar observation had been made with curare administered subcutaneously. However, when the alkaloid was administered *intravenously* a definite synergistic action was demonstrated (experiment 1, 3 and 4, table 2). The amount of dihydro- $\beta$ -erythroidine employed was approximately one-third of that necessary to produce complete paralysis in the cat. In the experiment in which the injection was made 20 minutes after the injection of pentobarbital, no distinct synergistic effect was noted, again indicating the importance of the time interval between the administration of the drugs. Prostigmine counteracted the peripheral action but did not inhibit the central action of the combined erythroidine-pentobarbital effect (experiment 3, table 2). This was made apparent by the state of drowsiness and muscular incoordination present in the animal after the administration of prostigmine. It should be noted that in some experiments the combined action of dihydro- $\beta$ -erythroidine and pentobarbital produced slight anesthesia in addition to paralysis. Such anesthesia was not observed in the curare-pentobarbital animals.

*C. QUININE ESTERS.* Since it had been shown by several investigators (6, 10, 11, 12) that quinine methochloride and quinine ethochloride possessed curare-like properties, it seemed of interest to study the action of these substances when combined with ether in mice and with pentobarbital sodium in cats.

*I. Action of quinine methochloride and quinine ethochloride with ether in mice.* Quinine methochloride and quinine ethochloride when injected subcutaneously

TABLE 2

*Dihydro  $\beta$ -erythroidine experiments in cats pretreated with pentobarbital sodium*

NO. OF EXPERIMENT	WEIGHT OF CAT IN KG.	PENTOBARBITAL SODIUM	INTERVAL BETWEEN PENTOBARBITAL SODIUM AND ERYTHROIDINE INJECTION	DIHYDRO $\beta$ -ERYTHROIDINE HBr	RESULTS
		mg./kg. (i.p.)	minutes	mg./kg. (i.v.)	
1	3.4	10	10	0.4	1 min. later—cat sleeping on side. 10 min. later—deep narcosis, complete muscle relaxation. 45 min. later—same. 1 hr. 20 min. later still in deep narcosis.
2	3.9	10	20	0.4	47 min. later—weak and not coordinated. Not sleepy.
3	2.4	10	13	0.4	Immediately lies on side, 5 min. later paralyzed, slight anesthesia, pupils small. 10 min. later—same. 13 min. later—i.v. injection of 0.1 mg. <i>prostigmine</i> . Cat stood up—walked unsteadily. 42 min. later—ran around, still unsteady in hind legs—some drowsiness.
4	2.4	10	5	0.3	5 min. later—tried to walk—very unsteady, 10 min. later—lies on side, sleepy, some anesthesia. 22 min. later—tried to walk—no longer sleepy.
5	3.0	—	—	0.4	2 min. later—hind legs weak—struggled to walk. 15 min. later—sat normally—alert. 75 min. later—normal.
6	3.6	—	—	0.4	Immediately—dyspnea. 10 min. later—normal. 50 min. later—quiet.
7	2.3	—	—	1.00	Immediate respiratory failure, dying. 1 min. later—0.25 mg. <i>prostigmine</i> i.v. 3 min. later—reacted to <i>prostigmine</i> . Still paralyzed. 20 min. later—walked normally.

into mice were ineffective at doses of 50–100 mg. per kg. When mice premedicated with these quinine esters (40 mg. per kg.) were exposed to an ether concentration of 0.25 cc. in a 1.5 liter bottle only a slight paralyzing action was observed. With 80 mg. per kg. of either ester a somewhat greater effect was demonstrated.

*II. Action of quinine methochloride and quinine ethochloride with pentobarbital sodium in cats.* Subcutaneous administration of these esters in doses as large as 17–25 mg. per kg. had no definite influence in increasing the paralyzing or narcotizing action in cats premedicated with pentobarbital. Better results were obtained with quinine methochloride injected *intravenously*. For example, the intravenous injection of small doses of this quinine ester ( $\frac{1}{4}$  of fatal intravenous dose) immediately paralyzed cats pretreated with pentobarbital sodium (table 3).

As in the curare and erythroidine experiments, premedication with the barbiturate is the most important factor in changing a refractory animal to one sensitive to paralyzing drugs. Whereas, immediate paralysis follows the administration of quinine methochloride to animals premedicated with pentobarbital, only light sleep without complete muscular relaxation occurs in animals premedicated with the quinine ester and 15–30 minutes later injected with the hypnotic. This is probably due to the disappearance of the quinine esters from the circulation into the tissues where they are partly transformed into inactive quinine compounds which are quickly excreted. This was demonstrated by an experiment in which a cat weighing 2.8 kg. was injected intravenously with quinine methochloride (2 mg. per kg.) and 30 minutes later received pentobarbital (10 mg. per kg.) intraperitoneally. Within 25 minutes the cat became somewhat sleepy and after one hour was very sleepy but at no time was there any evidence of a paralyzing action.

Quinine methochloride (1 mg. per kg.) and strychnos curare (0.2 mg. per kg.) simultaneously injected intravenously had no greater effect when combined with pentobarbital (10 mg. per kg. intraperitoneally) than did quinine methochloride and curare alone. Prostigmine counteracted the peripheral curare-like effect of quinine methochloride on the neuromuscular junction, but did not interfere with its action on cerebral synaptic transmission (experiment 2, 3, 4, table 3).

*D. ACTION OF QUININE SULFATE WITH PENTOBARBITAL SODIUM IN CATS.* The generally known depressant action of quinine salts on the striated muscles and the suggestion by Harvey (13) that this effect may be attributed to a curare-like action, led us to consider whether a combination of quinine salts with hypnotics would have an action similar to that of pentobarbital sodium combined with quinine esters. Experiments with quinine sulfate administered intravenously to cats premedicated with pentobarbital, however, showed no synergistic effect. Moreover, prostigmine had no counteracting effect on the action of this combination.

Apparently, unlike the quinine esters, quinine has no central curare-like synergistic action with anesthetics.

*E. ACTION OF MORPHINE SULFATE WITH CURARE-LIKE ALKALOIDS.* In previous experiments it was observed that combination of curare-like alkaloids with ether

TABLE 3

Quinine methochloride experiments in cats pretreated with pentobarbital sodium

NO. OF EXPERIMENT	WEIGHT OF CAT IN KG.	PENTOBARBITAL SODIUM	INTERVAL BETWEEN PENTOBARBITAL SODIUM AND QUININE METHOCHLORIDE INJECTION	QUININE METHOCHLORIDE	RESULTS
		mg./kg. (i.p.)	minutes	mg./kg. (i.v.)	
1	2.4	10	10	2.0	Immediately paralyzed not anesthetized. 15 min. later—unable to rise. 20 min. later struggled, unable to stand. 55 min. later—started to move. 70 min. later—walked almost normally.
2	3.7	10	7	2.0	Immediately paralyzed—lies on side. 3 min. later—some movements. 6 min. later—0.25 mg. <i>prostigmine i.v.</i> 1 min. later—struggled to walk. 15 min. later—staggered—rested frequently. 60 min. later—almost normal.
3	3.4	10	10	2.0	7 min. later—cat lies on side, some paralysis, not completely relaxed. 14 min. later—tried to walk. 15 min. later—0.25 mg. <i>prostigmine i.v.</i> , ran immediately, staggered. 7 min. later—lies on side. 20 min. later—attempted to walk. 80 min. later—walked normally.
4	1.8	10	10	2.0	No immediate additional effect. 20 min. later—on side, sleeping, paralyzed. 20 min. later—0.25 mg. <i>prostigmine i.v.</i> Cat immediately sat up but did not walk. 40 min. later—did walk—weak. 105 min. later—almost normal.
5	2.8	—	—	2.0	Not affected.
6	2.9	—	—	2.0 Quinine methochloride +0.4 Strychnos curare	Not affected.
7	2.1	—	—	5.0	Not affected.
8	3.0	—	—	8.3	Immediate respiratory paralysis.

or with pentobarbital sodium may considerably enhance the action of the curare drugs. From the theoretical as well as the practical point of view, it seemed important to determine whether pretreatment with morphine sulfate, which is generally used as a pre-anesthetic medication, either alone or combined with scopolamine would strengthen the action of the curare-like alkaloids. Morphine sulfate was, therefore, administered subcutaneously to mice (80 mg. per kg.) and to cats (3-6.5 mg. per kg.) and intravenously to rabbits in doses of 2.5-10 mg. per kg. After a short time small doses of the curare-like substance were administered either subcutaneously or intravenously.

The experiments in mice showed that pretreatment with morphine sulfate undoubtedly enhanced the action of d-tubocurarine chloride, dihydro- $\beta$ -erythroidine hydrobromide, and quinine methochloride since paralysis in the pre-medicated animals followed immediately the administration of sub-effective doses of these alkaloids. It was interesting to note that the stimulating effects of morphine on the brain and the spinal cord, indicated by restlessness and a tail reflex, disappeared simultaneously with the administration of the curare-like alkaloids and reappeared again only when they were inactivated either by destruction in the body or by excretion.

On the other hand, when mice were injected first with d-tubocurarine chloride (0.2 mg. per kg.) or with dihydro- $\beta$ -erythroidine hydrobromide (2.0 mg. per kg.) and 10 to 12 minutes later with morphine sulfate (80 mg. per kg.) no enhancement of action was apparent since the animals all exhibited typical morphine excitement and tail reflexes and no evidence of paralysis was noted. Apparently, therefore, these small amounts of curare and erythroidine are quickly inactivated in the mouse body. When either quinine methochloride or quinine ethochloride (80 mg. per kg.) were administered subcutaneously and, after an interval of 85 minutes, morphine sulfate (40 mg. per kg.) was injected, the mice failed to exhibit excitement but did show the typical tail reflex. In cats premedicated by the *subcutaneous* injection of morphine sulfate (3.5-6 mg. per kg.), *subcutaneous* injection of quinine methochloride (40-50 mg.) 5-8 minutes later failed to suppress the excitement produced by morphine.

In the series of experiments conducted in rabbits the injections of morphine and of the curare-like alkaloids were made intravenously. Only a few of these animals exhibited an immediate relaxing effect similar to that noted in cats receiving curare combined with pentobarbital sodium. In most of the animals the relaxation and sleep which occurred was delayed and seemed to be more the result of a belated morphine action rather than a synergistic effect of the morphine-curare combination. Also in these experiments prostigmine did not produce a definite counteracting effect. While it cannot be denied that a combined central morphine-curare or morphine-erythroidine action may be involved in these experiments, the present pharmacological analysis offers no definite evidence that morphine increases the paralyzing effect of curare-like alkaloids as does the combination of pentobarbital sodium with these substances. A similar doubtful result was observed when curare was combined with scopolamine or with morphine and scopolamine. The scopolamine-curare combination was ineffective. The

curare-scopolamine-morphine combination produced an immediate but very transient sleep.

Besides morphine and scopolamine, we used papaverine to determine whether it would enhance the curare action. It is known that the hypnotic effect of papaverine may be increased when combined with some sulfa drugs as well as in the case of certain barbiturates (Glaubach, 14). In our experiments, performed on rabbits averaging 3 kg. in weight, there was no narcotizing effect when strychnos curare (0.35 mg. per kg.) was injected intravenously seven minutes after an intraperitoneal injection of 0.15 gm. per kg. of papaverine hydrochloride. Similar negative results were noted when rabbits given an intravenous injection of d-tubocurarine chloride (0.017–0.033 mg. per kg.) and several minutes later a subcutaneous injection of 0.15 mg. per kg. of papaverine hydrochloride.

**DISCUSSION.** The above results are noteworthy in several respects. First they seem to show more exactly and quantitatively than clinical observations, the synergistic action of some anesthetics and curare or curare-like alkaloids. Clinical studies, mainly with ether, but also with avertin and with certain barbiturates, have shown an appreciable synergistic effect with d-tubocurarine chloride (2, 4, 15, 16, 17). Cyclopropane, ethylene or nitrous oxide are apparently quite lacking in this effect. Cullen (2) and Griffith (18) report, for example, that doses of Intocostin given to a patient under ether anesthesia had two or three times the effect of the same dose given to a patient under cyclopropane, ethylene, or nitrous oxide. Our experiments with mice treated with a combination of curare and ether confirm these clinical findings. They indicate, moreover, that this synergistic action may make it possible to produce the desired degree of anesthesia with appreciably smaller amounts of ether as well as alkaloid than would be required to produce an effect if the drugs were used alone.

Combining individually ineffective doses of pentobarbital sodium and curare, pentobarbital sodium and dihydro- $\beta$ -erythroidine or pentobarbital sodium and quinine esters enhanced the action of each and produced powerful and sometimes fatal effects; while combination of the curare-like substances and urethane, papaverine hydrochloride, quinine sulfate, or scopolamine chloride showed no evidence of synergistic action. Results of experiments in which morphine was combined with curare alkaloids indicated a definite synergistic action in mice; evidence of such action in cats or rabbits was not so conclusive. The most striking results were obtained when curare and the curare-like alkaloids were administered intravenously, subcutaneous injection being effective only in mice. In animals premedicated with curare-like alkaloids, the subsequent administration of hypnotics produced relatively little synergistic effect probably because of the rapid destruction or elimination of the former.

In cats injected intraperitoneally with pentobarbital sodium in ineffective doses, subsequent intravenous injection of curare or curare-like alkaloids produced complete muscular relaxation. Indeed, sometimes complete paralysis was obtained with as little as      of the amount of the uncombined alkaloid necessary to produce the same effect. Because the doses of anesthetics and alkaloids were clearly ineffective when administered alone the combined action represents a real

synergistic effect in which the anesthetics as well as the alkaloids are involved. These experimental observations are in accord with the report of Gray and Halton (4) in which the synergistic combination of d-tubocurarine chloride (15 mg.) with a minimal anesthetic dose of pentobarbital produced a completely anesthetized and motionless patient despite the fact that curare alone has no anagelsic action. It should be mentioned here that enhanced anesthesia was not observed in any of our curare treated animals. It was, however, noted in cats receiving dihydro- $\beta$ -erythroidine and pentobarbital sodium and in mice treated with quinine methochloride and morphine. The synergistic action pertained only to the alteration of muscle movements and muscle tonus; the sensory perception remained unchanged. These observations are in accordance with the report of Whitacre and Fisher (17) in which evidence of pain was noted in patients operated upon under complete curare paralysis and with loss of consciousness.

The knowledge that muscular relaxation sufficient for clinical purposes may be obtained by the combined action of small amounts of alkaloids and hypnotics seems to be of importance from a practical point of view. Our experiments show that when larger amounts of pentobarbital are used good muscular relaxation will be established with smaller amounts of the alkaloids. The total effect is, of course, dependent on the size of the doses and persistence of action of the drugs used.

The synergistic action of ether or the barbiturates with curare and curare-like alkaloids seems to be due more to a central curare action than to a peripheral one. The experiments of Auer and Meltzer (19) and Gross and Cullen (20) on isolated muscle-nerve preparations suggest that a peripheral action of ether or the barbiturates on the myoneural junction may be involved in the combined action of curare-ether and curare-barbiturate. However, many experimental and clinical facts have indicated a central as well as a peripheral site of action of these alkaloids. The action on the electrical brain potentials (5, 6, 7), the blocking of the sensory impulses which influences the respiratory center before the full development of its peripheral action (21), and finally the inducement of sleep and abrupt loss of consciousness by administration of curare (1, 10, 17) are examples of its central effect. Furthermore, the administration of curare by intracisternal injection or by way of the internal carotid artery caused a rise in blood pressure in cats with accelerated respiration, central vagal stimulation and prolonged local and generalized convulsions; paralysis of vasomotor and respiratory centers only occurred following prolonged action of larger doses (22).

The reason for the enhancement of curare activity by pretreatment with ether or pentobarbital is not clear. Alteration in the permeability of the brain tissues by premedication with anesthetics or hypnotics may be one of several factors involved. The fact, however, that curare paralysis is mediated through both its peripheral and central actions as well as by alteration of various spinal and cerebral reflexes, seems to exclude a simple explanation of the synergistic action with ether or pentobarbital sodium. These conditions are even more complicated since the action of ether and of barbiturates must be recognized as being to some extent peripheral as well as central (19, 20).

## SUMMARY

1. Premedication of mice and cats with small doses of ether, phenobarbital sodium, or pentobarbital sodium enhances the sensitivity of the animal to d-tubocurarine chloride, strychnos curare (Merck), dihydro- $\beta$ -erythroidine hydrobromide and quinine methochloride or quinine ethochloride but not to quinine salts. Sub-effective amounts of these curare-like alkaloids have a paralyzing effect on mice and cats so premedicated. The effective doses of curare and the curare-like alkaloids for the pretreated animals are  $\frac{1}{8}$  to  $\frac{1}{2}$  of the dose necessary to produce a similar paralyzing effect in untreated animals. There is a reciprocal relationship between the amounts of the alkaloids and hypnotics necessary to produce this synergistic effect.

2. Premedication with urethane, papaverine hydrochloride or scopolamine chloride produces no distinct sensitivity for the aforementioned curare-like alkaloids. Pretreatment with morphine increases the effect of these curare substances in mice, but produces only a doubtful effect in cats and rabbits.

3. *Subcutaneous* administration of curare and the curare-like alkaloids produced sensitization in mice, but not in cats. In this latter species *intravenous* injection of curare is highly effective.

4. Premedication with anesthetics (ether) or hypnotics (barbiturates) followed by injection of curare alkaloids seems to be justified from a practical point of view since it produces an increased sensitivity to the curare drugs and thus permits attainment of a degree of muscular relaxation which cannot be obtained with the same amount of curare alone.

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# THE EFFECTS OF VARIOUS CHEMICALS ON THE SURVIVAL OF FROG MUSCLE AND NERVE AFTER SOMATIC DEATH

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**INTRODUCTION.** The long survival of frog's excised muscle and nerve when kept in refrigerated Ringer solution (Sollmann, 1946) offers the opportunity to study slower and more prolonged actions than is usually attempted in experiments with excised organs. The primary object of the present investigation was to learn whether the processes of natural death of tissue may be influenced by chemical reagents, as they are by temperature; especially, whether they may be suspended or deferred, a question which opens some theoretical vistas, and which could have practical applications for instance in the transplantation of tissues. Brunquist, 1937, in a brief note reported the marked effects of temperature on the survival of the response of frog sartorius muscle when kept in Ringer solution, averaging 4.6 days at 25°C, 12 days at 13°C, 26 days at 5° to 6°C. It seemed conceivable that chemicals which affect metabolism, such as cyanide, or local anesthetics, might produce analogous results. Aside from this, it appeared interesting to observe the effects of chemicals when they act on the excised tissues for days instead of hours. In order to map out the field as a whole, thirty-two substances of various types were investigated as far as they appeared promising for this purpose.

**METHODS.** Nerve-muscle preparations of the arms and legs of frogs were dissected, sometimes fully, sometimes only superficially. The preparation of one side was placed as control in a beaker with about 50 cc. of a refrigerated dextrose-Ringer solution (per cent, NaCl 0.65; KCl 0.015; CaCl<sub>2</sub> 0.015; NaHCO<sub>3</sub> 0.02; MgCl<sub>2</sub> 0.01; dextrose 0.1). The contralateral preparation was placed in the same amount of the same solution containing the chemicals. The preparations were tested for excitability and stored in a refrigerator near 0°C. The tests and solutions were generally renewed three times per week. To check the effects on the nerve itself, leg preparations were suspended by the Achilles tendon from the stopper of small half-filled, wide-mouthed hottles so that only the nerve was immersed. This arrangement, suggested by Lahe, 1929, forms an adequate moist chamber.

Stimulation was applied through the ordinary platinum electrodes of a Harvard induction coil, activated by a dry cell of about 1½ volts, using three strengths of current, with the secondary at 12, 6, and 0 cm. To facilitate the handling of the data, the responses were graded arbitrarily from 0 to 6; and for further simplification as: *Fully excitable* (grade 6), good response at 12 cm.; *half-decline* (grade 3), no or slight response at 12 cm., fair response at 6 cm., good response at 3 cm.; and *inexcitable* (grade 0), no or practically no response at 0 cm. The responses were plotted against time on semilogarithmic paper, to permit interpolation, but it does not appear necessary to reproduce these details. Attention was directed especially to the *survival time*, the time which elapses until the preparation becomes inexcitable, as the simplest criterion of long-term action. It is conveniently expressed as a fraction, with the days in the drugged Ringer solution as numerator, and the days in unpoisoned solution as denominator, and this may be reduced to a decimal

TABLE 1

*Table of survival time quotients (drugged divided by undrugged preparations)*

CHEMICAL	DILUTION, 1:	SURVIVAL SHORTENED Q=<0.8		NORMAL RANGE Q=0.8-1.25		SURVIVAL PROLONGED Q=>1.25	
		Stimulation of					
		Nerve	Muscle	Nerve	Muscle	Nerve	Muscle
Sulfanilamide	1000	—	—	—	—	2.7	3.5
	5000	—	—	1.0	1.0	—	—
Sulfide, sodium	1000	—	—	—	—	2.25	1.8
	10,000	—	—	—	—	—	1.5
	50,000	—	—	—	0.93	—	—
Cyanide, sodium	1000	2 h. to a week		—	—	—	—
	5000	2 h. to a week		—	—	—	—
	10,000	—	—	—	—	1.8	1.8
	12,000	—	—	—	—	2.2	2.3
	25,000	—	—	1.2	1.2	—	—
	100,000	—	—	0.92	0.92	—	—
Phenol	1000	< 1 day		—	—	—	—
	3333	—	—	>11 days		—	—
	5000	—	—	1.0	1.0	—	—
	10,000	—	—	0.87	1.1	—	—
Hydroquinone	10,000	0.5	0.5	—	—	—	—
	20,000	—	—	0.87	0.88	—	—
Hydroquinone oxidized	10,000	—	—	1.0	0.8	—	—
Quinone	10,000	0.73	0.73	—	—	—	—
	20,000	—	0.75	1.0	—	—	—
	100,000	—	—	1.2	—	—	1.3
Tertiarybutyl catechol	1,000	<5 min.		—	—	—	—
	5,000	<3 days		—	—	—	—
	10,000	5 h. to 1 day		—	—	—	—
	20,000	0.7	0.7	—	—	—	—
2-6 Dinitrophenol	10,000	$\frac{1}{2}$ -1 $\frac{1}{2}$ hour		—	—	—	—
	50,000	0.2	0.3	—	—	—	—
	250,000	—	—	1.0	1.0	—	—
Acetanilid	1,000	0.5	0.6	—	—	—	—
	10,000	0.7	—	—	0.8	—	—
Procaine hydrochloride	1,000	<1 day		—	—	—	—
	5,000	—	—	1.	1.	—	—
	10,000	—	—	1.	0.9	—	—
Para-amino benzoic acid neutralized	100	—	—	—	1.0	1.9	—
	1,000	—	—	—	1.2	1.4	—

TABLE 1—Concluded

CHEMICAL	DILUTION, 1:	SURVIVAL SHORTENED Q=<0.8		NORMAL RANGE Q=0.8-1.25		SURVIVAL PROLONGED Q=>1.25	
		Stimulation of					
		Nerve	Muscle	Nerve	Muscle	Nerve	Muscle
Benzoate, sodium	100	1½ h.	6 h.	—	—	—	—
	200	6 h.	>2 d.	—	—	—	—
	300	<1 day	—	—	—	—	—
	500	—	—	1.2	1.2	—	—
Salicylate, sodium	100	<30 min.	<30 min.	—	—	—	—
	1,000	<1 day	<1 day	—	—	—	—
	5,000	0.6	0.4	—	—	—	—
	10,000	—	—	1.0	1.0	—	—
Thiocyanate, sodium	1,000	<1 day		—	—	—	—
	3,000	1-3 d.	3-5 d.	—	—	—	—
	5,000	—	—	0.8	0.8	—	—
Monoiodoacetate, sodium	3,000	<3 hours		—	—	—	—
	10,000	<2½ h.	<1 day	—	—	—	—
	30,000	<3 days		—	—	—	—
	50,000	0.5	0.4	—	—	—	—
Fluoride sodium (in iso- tonic NaCl)	5,000	0.25	0.67	—	—	—	—
	10,000	—	—	0.81	0.81	—	—
	30,000	—	—	1.0	1.0	—	—
Oxalate, sodium (in iso- tonic NaCl)	3,000	0.5	—	—	0.9	—	—
	10,000	—	—	0.87	0.87	—	—
Methylene blue	1,000	0.61	0.72	—	—	—	—
Thiourea	100	—	—	1.2	1.2	—	—
Urethane	33	<1 day		—	—	—	—
	500	—	—	1.0	1.0	—	—
Chloral Hydrate	1250	(0.2 for half-decline)		—	—	—	—
	2000	0.75	0.75	—	—	—	—
Mecholyl chloride	3000	—	—	1.0	1.0	—	—
Physostigmine salicylate	5000	0.5	0.5	—	—	—	—
	10,000	—	—	1.0	1.0	—	—
Vitamins: Ascorbic acid Nicotinic acid Pantothenate calcium Pyridoxine Riboflavin Thiamine hydro- chloride	10,000	—	—	1.0	1.0	—	—
	100,000	—	—	1.0	1.0	—	—
Insulin 200 units in 100 cc.	—	—	—	1.0	1.0	—	—
Thyroid, dried	1 grain in 50 cc.	—	—	1.0	1.0	—	—

quotient. Survival time is considered as shortened if the decimal quotient is less than 0.8; as prolonged if it is greater than 1.25; quotients between 0.8 and 1.25 are considered as normal variation.

Decline of the response of muscle to direct stimulation proves of course, that the muscle itself is depressed. If the response to nerve stimulation declines more than that to muscle stimulation, this shows that the nerve or the synapse is depressed; but this cannot be affirmed if muscle and nerve stimulation run parallel. In such case, the distinction must be made by immersing only the nerve, as in the arrangement which has been described.

The loss of excitability is usually accompanied by rigor; and if the preparation has been kept for some days, by the gradual development of turbidity and putrid odor in the solutions. Dr. Randall L. Thompson confirmed that this turbidity is due chiefly to bacteria, principally to a member of the *Proteus* group, with possibly two or three other bacteria in smaller numbers. This raises the question whether death is due to the bacteria, or whether bacteria develop because the tissue is dead, or whether there is an interplay of these two factors, as is most likely. The question could be resolved only by maintaining asepsis which would be difficult. The non-drugged contralateral preparation furnishes a measure of control; material hastening death may fairly be ascribed to direct injurious action of the drug on the tissue, since none of the agents that produced this effect are known to stimulate bacterial growth. Delayed death is more difficult to interpret, since so many substances have bacteriostatic action. This holds true for all the substances that delayed dying in these preparations, so that it is not possible to affirm that any of the substances that were tried delay dying by direct action on the tissue.

**RESULTS.<sup>1</sup>** The synopsis of the survival time in the accompanying tabulation shows the concentrations which were found to shorten survival for indirect and for direct stimulation; those which had no material effect, and those which deferred death. It does not seem feasible, to include in the table data on partial loss of excitability, or immersion of the nerve alone, or the development of rigor or turbidity or recovery in Ringer solution, without making this too complicated. It is, therefore, necessary to duplicate the data in the discussions.

**Discussion. Sodium Cyanide.** The original motivation of these attempts, to prolong the life of excised tissue by chemical slowing of their metabolism, was furnished by an observation on a reversible suspension of the development of the fertilized ova of *Fundulus heteroclitus* by suitable concentrations of cyanide (Sollmann, 1906): If the ova, from the four cell stage to the time when heart and blood vessels are formed, are placed in sea water containing potassium cyanide 1:1000, their further development is promptly arrested, but the cells do not die for two or three days: if they are transferred to pure sea water or to more dilute cyanide, at any time in the interval, development resumes after a latent period of one or two days, the embryo hatching normally, but two to four days after the control. It was thought *a priori* that survival of muscle and nerve might be similarly prolonged by suspending their metabolism with cyanide. This expectation was disappointed. The excitability of muscle and nerve may be abolished by cyanide of sufficient concentration, but this depression does not appear to be reversible. Survival may be considerably prolonged by dilute cyanide solutions; however, this is not based on reversible suspension of excitability, but on bacteriostatic action.

<sup>1</sup> I am much indebted to Dr. José J. Estable for assistance in some of the later experiments.

*Sodium Cyanide* has opposite actions according to the concentration; 1:5000 and higher are toxic to both muscle and nerve; but dilutions of 1:10,000 to 1:12,500 prolong the survival time. With 1:10,000, response to nerve and to muscle stimulation survived 32 vs. 18 days (Q. 1.8); with 1:12,500 responses to nerve stimulation persisted for 20 vs. 9 days (Q. 2.2), to muscle stimulation 22 vs. 9 days (Q. 2.3); 1:25,000 was practically indifferent, both muscle and nerve remaining excitable for 15 vs. 13 days (Q. 1.2); 1:100,000 gave Q. 0.92. The 1:12,500 concentration does not prevent *rigor*; it is doubtful whether it delays this beyond the change of excitability.

The prolonged survival in cyanide parallels the effect on bacterial turbidity, and the deferment of death is presumably due to inhibition of bacterial putrefaction, as with sulfanilamide and sulfide. The delay of turbidity is noticeable even with 1:100,000 (10 vs. 7 days, Q. 1.4); more marked with 1:25,000 (16 vs. 2 days, Q. 8.); very great with 1:10,000 (>33 vs. 10 days, Q. > 3.3) and 1:3000 (23 vs. 2 days, Q. 12.). It appears therefore that cyanide checks bacterial growth in dilution of 1:25,000, perhaps even 1:100,000, when refrigerated. Cyanide is not usually considered a potent bactericide, but it is more highly effective as bacteriostatic. This was confirmed by Dr. Thompson on bacterial cultures: at room temperature, 1:5000 completely inhibited the four forms; 1:10,000 and 1:25,000 generally prevented growth, but not always. Refrigerated cultures were all completely inhibited by 1:25,000.

*Depressive Concentrations of Cyanide:* The concentration of 1:1000 abolishes response to nerve and to muscle stimulation, sometimes in two hours, sometimes only after several days but in less than a week. The response to nerve stimulation disappears rather more promptly than that to direct muscle stimulation, indicating depression of the nerve or synapse, as well as of muscular contractility. Immersion of the sciatic nerve in 1:5000 abolishes its excitability in  $\frac{1}{2}$  hour to several days, the latter in a third the time of the controls. The nerve fibers are therefore depressed and killed directly. Transfer to unpoisoned Ringer solution for up to nine days did not improve excitability of nerve that had been abolished by immersion in 1:5000 for 3 and 5 days; nor did it improve the half-depressed excitability of muscle immersed in 1:1000 for 7 days; It did rather questionably restore slight response in muscle whose response had been abolished by 1:1000 for 1 to 7 days. The restoration is therefore practically nil. The total survival time in these slightly restored muscles was about the same as in the unpoisoned Ringer control preparation; rather shorter on the average.

Cyanide in concentration of 1:1000 to 1:10,000 produces peculiar changes in the appearance of muscle. This becomes pellucid and seems swollen, "sulzig", after one or two days and remained so till the experiment was discontinued in 17 days. The appearance was not changed by transferring to Ringer solution for 9 days. This change is not the cause of the toxicity, for the muscle remained fully excitable for 17 days in 1:10,000 solution, although it had become pellucid, the beginning of this change being noted in six days.

OTHER AGENTS WHICH PROLONG SURVIVAL; SULFANILAMIDE, SODIUM SULFIDE, PARA-AMINO BENZOATE. These and cyanide were the only agents that in suitable concentrations, prolonged survival. This was paralleled by inhibition of bacterial turbidity and is presumably due to their bacteriostatic effect.

*Sulfanilamide*, 1:1000, delayed deterioration and prolonged survival more than any other agent, 27 vs. 10 days for nerve stimulation (Q. 2.7), 40 vs. 12 days for muscle (Q. 3.5). Full excitability of muscle and nerve was maintained for 15 vs. 10 days (Q. 1.5). Survival was not prolonged by 1:5000 (17 vs. 17 days, Q. 1) for both nerve and muscle; but full excitability lasted perhaps somewhat longer than in the control, 12 vs. 10 days (Q. 1.2), for both nerve and muscle.

Sulfanilamide similarly delayed the development of bacterial turbidity in the solution. The Ringer control was slightly turbid in 5 days, turbid in 8 days; but the fluid of the 1:1000 sulfanilamide preparation remained clear for 30 days, becoming turbid in 37 days, with putrid odor. Dr. Thompson confirmed that sulfanilamide 1:1000, added to cultures of four test organisms including the proteus strain, inhibited growth for 90 hours; two of the four showed growth in 114 hours. The 1:5000 dilution also showed some bacteriostatic action; the solution bathing the muscle remained clear for 15 days, while the Ringer control became fairly turbid in 11 days.

The development of rigor mortis parallels the depression of excitability. In the 1:1000 solution, the muscles appeared flexible for 19 days, semi-rigid in 21 to 26 days, more rigid in 28 days.

*Sodium Sulfide*, 1:1000, considerably prolonged the survival for nerve (9 vs. 4 days, Q. 2.25), and muscle (11 vs. 6 days, Q. 1.8). Muscle survival was also prolonged in 1:10,000 (9 vs. 6 days, Q. 1.5). No effect was apparent with 1:50,000 (Q. 0.93) and 1:100,000 (Q. 1.05). Turbidity went closely parallel; in 1:1000 the fluid remained clear for more than 12 days, as against 4 days in the control. In 1:10,000 turbidity began in 8 vs. 6 days, Q. 1.3; in 1:50,000 in 7 vs. 7 days, Q. 1.0. The prolongation of survival is therefore attributable to bacteriostatic action.

*Para-aminobenzoic Acid (PABA)* was also found to prolong survival apparently by a bacteriostatic effect, although it had been expected to hasten death in analogy with benzoate and salicylate.

Neutralized with sodium bicarbonate, 1:100 does not depress the excitability of muscle and nerve, but prolongs the survival of the immersed sciatic nerve for 11 vs. 6 days, Q. 1.9. The PABA solution remained clear at the end of the 11 days, while the control was turbid on the sixth day. Immersed muscle survived as the control, 13 vs. 13 days, Q. 1. In 1:1000 the survival was also somewhat prolonged, 19 vs. 14 days, Q. 1.4 for nerve; 23 vs. 20 days, Q. 1.2 for muscle; the PABA solution being definitely less turbid than the control in 14 days.

**AROMATIC ANTISEPTICS WHICH SHORTENED SURVIVAL: HYDROQUINONE, QUINONE, TERTIARY BUTYL CATECHOL, PHENOL, 2,6-DINITROPHENOL, ACETANILID.** These agents were chosen as representing various types of antibacterial action in the aromatic group. However, they proved more injurious to the muscle than to the bacteria. The lowest concentrations that had any effect shortened the survival of response to muscle and nerve stimulation, in about equal degree, and also in the instances (hydroquinone) where the nerve alone was immersed. They are, therefore, directly toxic both to nerve and to muscle.

Concentrations of 1:1000 are acutely fatal with all except acetanilid, abolishing excitability in less than a day, generally within two hours, the tertiary butyl catechol even within five minutes. Acetanilid shortened survival time to 7 vs. 14 days, Q. 0.5.

Phenol gave normal survival in 1:5,000, (15 vs. 13 days) and probably in 1:3,333 (11 days). 1:10,000 shortened the survival of all except phenol: hydroquinone 1½ to 5 days; oxidized hydroquinone, 5 vs. 14 days, Q. 0.3; quinone less than one to 7 vs. 10 days; tertiary butyl catechol, 1 day; dinitrophenol 1 hour to less than 3 days; acetanilid 4 vs. 8 days, for nerve stimulation; Q. 0.5; 6 vs. 9 days, Q. 0.5 for muscle, Q. 0.67; phenol 12 vs. 14 days, Q. 0.86 for nerve, 17 vs. 16 days, Q. 1.1 for muscle. 1:20,000 shortened survival in hydroquinone to 11 vs. 16, Q. 0.69; in quinone to 5 vs. 9, Q. 0.55; and in tertiary butyl catechol to 10 vs. 13; Q. 0.77. 1:50,000 shortened survival in dinitrophenol to less than 3 days, the control surviving to 8 days, Q. 0.5. With all the other drugs survival in this concentration equaled the control.

The ranking of toxicity therefore appears to be in the order of: Dinitrophenol 2,6> tertiary butyl catechol> quinone> hydroquinone> acetanilid> phenol. *Acute and*

delayed fatality and shortened survival time grade into each other so smoothly that they are probably mere degrees of the same process; and this holds true for muscle and nerve, and for all these drugs, so that the action is probably analogous; although it may be remembered that there is not much opportunity for conspicuous variations in the process of dying.—*Transfer to unpoisoned Ringer's solution* was ineffective when the excitability had been lost in tertiary butyl catechol 1:10,000 and dinitrophenol 1:50,000, and this even when the transfer was made in 1½ hours. It was also generally ineffective for hydroquinone, but in exceptional instances there was partial restoration, as after quinone 1:10,000 for one day, when Ringer solution restored the response from zero to half, in both nerve and muscle. The total survival of the recovered preparations was shorter than for the control, 14 vs. 17 days, Q. 0.8. Altogether, reversal is none or slight.

The development of *muscular rigor* generally paralleled the depression and loss of contraction response; the muscle becoming "semi-rigid" when the response level had dropped to half, and "stiff" when the response was lost. Precipitation of proteins, which may be produced by some of these agents, therefore does not appear to hasten the rigor beyond that usual to dying tissues; nor was precipitation as such the cause of the loss of excitability, for with the more concentrated solutions the muscle may become inexcitable while it is still flexible.

*Brown discoloration* of the muscle occurs especially with hydroquinone, fresh and oxidized, and with quinone. These solutions turn brown on standing, and the muscle gradually takes on a deeper chocolate tint. With hydroquinone 1:10,000 this begins within three hours, is a light umber in a day, and darkens progressively through the six days of observation. The brown is almost as deep with 1:50,000, lighter but still marked with 1:100,000.

The nerve takes on little if any stain, even in 1:10,000. Some other aromatic reagents, such as salicylates and paraamidobenzoate, produce similar brown coloration of the solutions and muscle, not of the nerve.

*Bacterial turbidity* was checked effectively by the concentrations that shortened the survival of the muscles (Q. less than 0.8). The solutions were usually clear when the response of the muscles had become very weak, in hydroquinone and in quinone 1:10,000 and 20,000; dinitrophenol 1:50,000; acetanilid 1:10,000 (without counting those in which death occurred in less than two days, and in which there would not have been time for bacterial growth). Some turbidity occurred with oxidized hydroquinone 1:10,000, and tertiary butyl catechol 1:20,000, but in both cases materially slower than in the controls. Dr. Thompson confirmed that phenol, quinone, and hydroquinone inhibit bacterial growth at refrigerator temperature, in dilutions comparable to the higher concentrations used in the frog experiments, taking account of the lower temperature used for the latter. The ratio of bacteriostatic potency varies for different organisms; referred to phenol, quinone was 20 times as potent for staphylococcus aureus, but only 0.6 to 2.5 times for Escherichia coli. Hydroquinone was about twice as potent as quinone against Escherichia.

Lahe, 1929, reported that the toxicity of hydroquinone for frog sciatic nerve is materially accelerated by conditions that favor the oxidation, especially by alkalinity. In leech preparations (1930) he found it twenty times more potent in alkaline than acid solutions. In the present experiments, the toxicity of quinone and of fresh and aged hydroquinone solutions were of the same order of magnitude. It is therefore probably the maintenance of an equilibrium of the oxidation stages, which interferes with the oxidative metabolism of the tissues, somewhat as postulated by Lahe.

**PROCAINE HYDROCHLORIDE.** Temporary and reversible depression of excitability suggested itself as another possible approach for prolonging survival. This was attempted with procaine; but it was not successful. Reversibility of the procaine depression of nerve was found to obtain only for rather narrow limits of concentration and especially of time, far less than the normal survival

time. Concentrations that have any effect at all, produce progressive depression of response to stimulation, and so shorten the survival time. It was also found that the response of the muscle to direct stimulation went parallel to the depressed response for nerve stimulation, almost quantitatively as well as qualitatively. Procaine, therefore depresses muscle directly, about at the same rate as it depresses the excitability of the nerve fibers. The action on nerve fibers was confirmed by immersion of the sciatic nerve exclusive of the muscle.

*The speeds and degree of depression* increases with the concentration: Full excitability of muscle and nerve with secondary coil at 12 cm. is preserved in 1:250 concentration for 20 to 45 minutes; in 1:500 for 75 to 100 minutes; in 1:1000 and 2,500 for between 3½ hours and one day; it is not shortened by 1:50,000 and 10,000. "Half-decline," with little or no response at 12 cm., but good or fair response at 6 cm., is reached in 45 minutes with 1:250; 140 minutes with 1:5000; 5 hours with 1:4000. *Inexcitability*, with coil at 0 cm., is reached within one day with 1:250 to 2,500. The survival time in 1:5000 is the same as in Ringer controls, 12 days in one pair, 15 days in another.

*Reversibility by transfer to unpoisoned Ringer solution* seems to depend on the concentration and time of contact more than on the degree of depression: With 1:1000 only partial recovery occurred after one day in the procaine solution, none after four days; With 1:500, good recovery occurred after 140 minutes, none after a day of contact. In 1:250 good recovery occurred after one hour.

These experimental results appear in conflict with the current clinical concept that procaine is a freely reversible specific depressant of nervous tissue. They appear to class procaine with so many of the other chemicals used in these studies, as a general protoplasmic depressant, rather than as a specific depressant of nervous tissue. However, the clinical reversibility would be expected from the relatively brief contact. The clinical specificity for nervous tissue may be attributed to the manner of its application, which is generally aimed to give the highest concentration in the nervous tissue which it is desired to depress. When it is injected into the muscle, for the relaxation of proprioceptive tonus in muscle spasm, or for the "pseudohernia" experiment of guinea pigs, its action may be directly on muscle, as also the suppression of cardiac fibrillation.

**OTHER AROMATIC ACIDS.** *Salicylate of sodium*, in higher concentrations, is known to produce peculiar modifications in muscle. It proved about equally toxic to nerve and muscle, rather more so than procaine, being injurious in all concentrations tried, somewhat even in 1:100,000.

Excitability was lost within half an hour in 1:100; within two hours in 1:250, with either nerve or muscle immersed; in 1 to 3 days with 1:1000, in 7 vs. 9 days, Q. 0.77 in 1:10,000. Transfer to Ringer solution produced some restoration even when responses had been abolished by immersion in 1:250 or 1:500 for two hours; but the recovery was only partial (grade 2 for nerve, 3 for muscle). No restoration occurred after one day's immersion in 1:1000. The loss of excitability was not the consequence of rigor; in 1:250 the muscle was still flexible when it was totally inexcitable; in 1:10,000 rigidity developed as in the control. The muscle is stained brown, the nerve not. *Bacteriostatic action* was insignificant; 1:500 did not inhibit bacterial turbidity.

*Benzoate of sodium*, which is so closely related to PABA, is very much more toxic to muscle and nerve.



In 1:100 solution, with only the sciatic nerve immersed, full response was obtained in 11 minutes, half decline in an hour, no response in 22 hours. When muscle as well as nerve was immersed, response for nerve stimulation had declined to half in half an hour, for muscle stimulation, in one and a half hours. No response was obtained from the nerve in one hour, from the muscle in three hours. In 1:500 solution, the immersed sciatic nerve was fully excitable in 1½ hours, inexcitable in 3 hours. Muscle and nerve immersion reduced the response of both to half within a day. Transfer to Ringer solution did not restore the excitability of sciatic nerve rendered inexcitable by immersion in 1:100 for three hours.—In 1:333, full response was obtained from nerve and muscle in one day, 1:500 and 1000 had no effect, the survival being the same as with the control.

*Summarizing the Aromatic Acids*, the salicylate and benzoate appear to act qualitatively like procaine, and like the phenols and quinones. Balancing the degree and speed of action with various concentrations, and using hydroquinone as unit, the *relative potency* is as follows: salicylate,  $\frac{1}{4}$  to  $\frac{1}{2}$ , mode  $\frac{1}{4}$ ; procaine  $\frac{1}{10}$  to  $\frac{1}{2}$ , mode  $\frac{1}{4}$ ; phenol  $\frac{1}{10}$  to  $\frac{1}{5}$ , mode  $\frac{1}{7}$ ; benzoate  $\frac{1}{20}$  to  $\frac{1}{2}$ , mode  $\frac{1}{2}$ . Paraaminobenzoate and sulfanilamide are not toxic in the highest concentrations that were tried.

**CATALYTIC POISONS.** *Sodium Thiocyanate, Monoiodoacetate, Fluoride and Oxalate.* These agents are grouped together as they have specific toxic actions on muscle metabolism and may be presumed to have similar actions on nerve.

*Thiocyanate* is known to produce twitching and contraction, of nervous and muscular origin, by immersion in two per cent solution. The more dilute solutions were used in the following experiments proved toxic to muscle, less so, if at all to nerve. This and the monoiodoacetate are the only substances among those tried, which act materially less on nerve than on muscle.

Immersion of muscle in 1:1000 solution decreased somewhat the response to direct stimulation within an hour, and abolished it between one and two days, the muscle becoming stiff. Sciatic nerve alone immersed in 1:1000 solution remained fully excitable for five days, after which the muscle went into rigor. Muscle immersed in 1:5000 remained fully excitable for five days, but declined to half by the eighth day and was inexcitable and rigid on the twelfth day, when the control muscle was still fully excitable. With 1:10,000 the muscle was fully excitable on the eighth day, declined to half on the eleventh day and still gave fair response on the thirteenth day. The toxicity of thiocyanate is of the order of salicylate, but its development is considerably slower, and it does not involve nerve significantly.

*Monoiodoacetate* has a relatively high acute toxicity, somewhat more for muscle than for nerve.

The response of muscle immersed in 1:3000 solution declined to three-fourths in  $\frac{1}{2}$  hour, to zero in 2½ hours. When nerve alone was immersed, its response declined to half in  $\frac{1}{2}$  of an hour, to a third in 2½ hours. In 1:10,000, muscle response declined to half within three hours, to zero within a day. Nerve alone immersed was fully excitable in two days, inexcitable in five days. In 1:50,000, muscle becomes inexcitable within three days. In 1:50,000, muscle was fully excitable in five days, inexcitable in eight days. The control muscle was fully excitable in eight days, half decline in twelve days, inexcitable in fifteen days. The total survival time was 8 vs. 15 days, Q. 0.5. Even in 1:100,000, the survival time was 5 vs. 10 days, Q. 0.5. Transfer to Ringer solution did not restore excitability after this had been abolished, in the muscle by three days in 1:30,000, in sciatic nerve by five days in 1:10,000. Development of rigor went parallel to the loss of excitability. Muscle

in 1:10,000 for 1½ hour, with somewhat more than half response, was still flexible; in 2½ hours with half response, it was semi-rigid; in 5 hours it was rigid and inexcitable.

*Fluoride of Sodium* (as also Sodium Oxalate) was dissolved in 0.7 per cent of sodium chloride, and compared with this instead of Ringer's solution. A peculiar feature was the relatively slow development of the toxic effects.

Even 1:5000 was not acutely toxic to muscle (although 1:20,000 produced temporary twitching); but it reduced the response to half in four days and abolished it within seven days. Sciatic nerve immersed alone was fully excitable in five days and then became totally inexcitable by the seventh day. Muscle immersed in 1:10,000 appeared fully excitable in two days, when it was transferred to unpoisoned Ringer solution. Its response then declined to ¾ on the fourth and seventh day, and to negligible on the eleventh day; the control muscle, kept in Ringer solution from the start, was fully excitable on the seventh day, half on the eleventh and fourteenth day, inexcitable on the sixteenth day. Notwithstanding the transfer from the fluoride to Ringer solution before deterioration was evident, the survival had been shortened to 12 vs. 15 days, Q. 0.8. With 1:20,000 the deterioration closely paralleled the control, with half decline in eight days, and inexcitability in twelve days. *Rigidity* appeared somewhat greater than the depression. In 1:5000, the muscle was semi-rigid in two days, when the response was still nearly maximal. *Turbidity* was not inhibited by 1:5000.

*Sodium Oxalate* in 0.7 per cent NaCl, shows the same delayed toxicity as the fluoride, but is less toxic.

In 1:3000, response declined to half in one day, to a third in five days, to nearly zero in eight days. In 1:10,000 the response was normal in 6 vs. 6 days, Q. 1; half in 9 vs. 11 days, Q. 0.8; zero in 13 vs. 15 days, Q. 0.8.

*Summarizing this group*, the toxicity declines in the order of monoiodoacetate, thiocyanate, fluoride and oxalate. The first two agents are peculiar in depressing nerve materially less than muscle; fluoride and oxalate are distinguished by the delayed development of their toxicity.

METHYLENE BLUE, THIOUREA, LIPOLYTIC AGENTS. URETHANE, CHLORAL HYDRATE. CHOLINERGIC DRUGS: MECHOLYL, PHYSOSTIGMINE. *Methylene Blue* was slowly toxic to muscle, less so to nerve. The highest concentration which was tried, 1:1000, did not impair the excitability of muscle for five days, then it declined to half by the seventh day and zero on the thirteenth day, when the control was fully excitable. The survival time was 13 vs. 18 days, Q. 0.7. Sciatic nerve alone immersed was fully excitable for 7 vs. 10 days, Q. 0.7; its total survival was 12 vs. 12 days, Q. 1.

*Thiourea*, 1:100, produced no deleterious effect. Full excitability persisted for 12 vs. 10 days, Q. 1.2; survival time was 18 vs. 16 days, Q. 1.2. This somewhat prolonged survival time is paralleled by some bacteriostatic action, the thiourea remaining clear for 19 days, while the control was slightly turbid on the fourteenth day and turbid on the seventeenth day.

*Urethane* depressed excitability of muscle promptly in 1:33, somewhat more slowly in 1:100, presumably by lipolytic narcosis. With 1:33, the excitability fell to half within thirty minutes, to a third within two hours, to zero within a day. In 1:100 excitability was full in two hours, declined to half in a day. The

responses to indirect stimulation declined similarly, so that the nerve was not affected more than the muscle.—*Transfer to Ringer solution* gave complete recovery from half-decline after one day in 1:100; practically no recovery from inexcitability after one day in 1:33.

*Chloral Hydrate*, 1:1250, did not impair excitability to direct and indirect stimulation in four hours, reduced it to half in three days, as against fifteen days for control, Q. 0.2; and abolished response in fifteen days. In 1:2000, unimpaired excitability persisted for eight days, declined to half in 12 vs. 14 days, Q. 0.85, and was abolished in 15 vs. 20 days, Q. 0.75. *Turbidity* was somewhat delayed.

*Cholinergic Agents* have little effect on survival time, none with physiologic concentrations.

*Mecholyl Chloride*, 1:3000 maintained full excitability for five days, with only slight decline in seven days. The survival time in 1:3000 to 12,000 was 16 vs. 16 days, Q. 1.

*Physostigmine Salicylate*, 1:5000 and 1:10,000, decreased the response but little in five days. *Survival time* was practically as for the control.

**VITAMINS.** *Ascorbic acid*, *nicotinic acid*, *pantothenate (calcium)*, *pyridoxine*, *riboflavin*, and *thiamine hydrochloride* were used in concentrations of 1:10,000 and 100,000. None had any effect whatever on the response to muscle and nerve stimulation, nor on the survival time.

**METABOLIC HORMONES.** *Insulin*: 200 units added to 100 cc. of Ringer's solution had no effect on response or survival time, 9 vs. 9 days, Q. 1.

*Thyroid*: A Burroughs, Welcome one grain tablet added to 50 cc. of Ringer's solution, did not alter the survival time, 10 vs. 10 days, Q. 1.

#### SUMMARY AND CONCLUSIONS

Prolonged actions of chemical agents on the survival time of excised muscle-nerve preparations of frogs were observed by immersing the preparations, or only the nerve, in a dextrose Ringer solution, kept near 0°C, until they ceased to respond to faradic stimulation; comparing the course in contra-lateral preparations with and without the chemical. The following substances which appeared interesting for various reasons were included:

Sulfonamide, sulfide, cyanide.

Hydroquinone, quinone, tertiary butyl catechol, phenol, 2,6-dinitro-phenol, acetanilid.

Procaine.

Para-aminobenzoate, benzoate, salicylate.

Thiocyanate, monoiodoacetate, fluoride, oxalate.

Methylene blue, thiourea, urethane, chloral hydrate, mecholyl, physostigmine.

Vitamins: Ascorbic acid, nicotinic acid, pantothenate, pyridoxine, riboflavin, thiamine hydrochloride.

Insulin, thyroid.

Prolongation of survival time was obtained only by substances that have a high degree of bacteriostatic action in concentrations that are not toxic to the

muscle and nerve; markedly with sulfanilamide, sulfide, sufficiently dilute cyanide, and somewhat with para-aminobenzoate.

Higher concentrations of cyanide shorten survival; and this was also the result with all the remaining substances that had any effect at all, in the concentrations used.

With the milder degrees of the injurious action, the excitability declines progressively for days, faster than in the control preparations. With the more toxic agents this grades smoothly into the acute toxicity, indicating that the rapid and slow affects are degrees of the same process.

For most agents, including procaine, the deterioration of nerve and of direct muscle response go closely parallel. None acted on the nerve materially more than on the muscle; thiocyanate, and to a less degree monoacetate acted materially less on the nerve than on the muscle.

The action of procaine on nerve does not appear specific, but rather a manifestation of general protoplasmic toxicity. Its apparent clinical specificity is presumably due to the location of its application. It is reversible only within a limited zone. Its action is almost equalled by salicylate and benzoate, while para-aminobenzoate prolongs survival and is not depressant even in one per cent concentration. Thiourea is also not depressant in this dilution.

Hydroquinone and quinone, dinitrophenol and tertiary butyl catechol are injurious in high dilutions.

Survival time was not modified by the vitamins in concentrations of 1:10,000 to 100,000; nor by insulin or thyroid; nor by mecholyl 1:3000, or physostigmine 1:5000. It was somewhat shortened by methylene blue 1:1000.

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# THE EFFECT OF DRUGS ON THE CONTRACTILE FORCE OF A SECTION OF THE RIGHT VENTRICLE UNDER CONDITIONS OF AN INTACT CIRCULATION

## MEASUREMENT OF ISOMETRIC SYSTOLIC TENSION BY MEANS OF CALIBRATED SPRINGS ATTACHED TO MYOCARDIOGRAPH LEVERS<sup>1</sup>

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**INTRODUCTION.** The method described here was developed for the purpose of obtaining a numerical figure indicative of the contractile force of a section of the myocardium under conditions of an intact circulation. The procedure consists essentially in the addition of graduated loads to the movable lever arm of the Cushny myocardiograph typically attached in the open-chest dog preparation. The graduated loads were obtained by stretching a calibrated metal spring directly attached to the string joining the kymograph writing lever and the myocardiograph lever, the pull of the spring being in opposition to the pull of systolic contraction. The spring tension necessary to damp the writing stroke to near extinction was taken as an end-point indicative of the contractile force of the myocardium. This critical load, or isometric systolic tension, expressed in grams, has been found to be reasonably constant and reproducible in a given preparation and it reflects with sensitivity the effects of stimulant or depressant drugs.

The lever system of the Cushny myocardiograph is relatively heavy as compared with other recording systems and this has justified, to some extent, the impression that amplitude of lever stroke is an index of contractile force. In the case of many drug effects, amplitude of lever stroke undoubtedly reflects the true qualitative changes. Stroke amplitude and isometric systolic tension, as determined here, do not, however, show consistently parallel changes and in some of our experiments showed distinctly opposite directions of change.

These changes in isometric systolic tension produced by drugs may, in some cases, be due almost exclusively to a primary effect on myocardial contractility. In other cases, the measured changes in contractility may represent a composite of influences in which primary or direct myocardial effects are only a minor component. These influences may include those due to sympathetic stimulation, to alterations in venous return or diastolic tension, in coronary flow, in pulmonary arterial pressure and in blood composition. These factors are listed here in what is estimated to be the approximate order of their prominence among the group of drug responses obtained in these experiments. In subsequent experimental

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work some of these factors may be excluded or measured simultaneously and correspondingly discounted. In this report, however, the results, subject to these composite influences, are presented for the value they may have in characterizing this method for measurement of myocardial contractility under a special set of conditions. The method has suitability for some particular purposes in that it provides a direct correlation of contractile force with blood pressure changes and with time and dosage variations in the whole animal.

The method has evident relationship to that described by Cattell and Gold (1) and subsequently used by Krop (2), Weeks and Holck (3) and White and Salter (4). In this latter, determinations are made of the tension developed during isometric contractions of isolated strips of papillary muscle which are stimulated by an intermittent electric current of fixed rate and voltage. Other methods of measuring effects of cardiotonic drugs may be indicated by citing some of the more significant publications. Francois-Franck (5) was one of the first to determine effects of drugs on intraventricular pressures and he particularly described the increased pressures produced by digitalis and strophanthin. Subsequently Gottlieb and Magnus (6) and Magnus and Sowton (7) introduced balloons into the ventricles of isolated mammalian hearts and, in the latter report, digitalis was described as doubling the isometric intraventricular pressure. DeHeer (8) made similar observations with mammalian hearts *in situ*. Wiggers and associates (9, 10), also working with open-chest dogs, analyzed the changes in intraventricular pressure curves produced by epinephrine and digitalis and found basic similarities including increased maximal pressures and increased gradients of pressure development. Bodo (11) has described the effects of various cardiovascular drugs on tracings obtained with the Henderson cardiometer. Krayner and associates (12-16) have made extensive studies of the effect of drugs on the efficiency of heart-lung preparations under conditions of measured changes in venous inflow. The effect of drugs on oxygen consumption of the heart muscle and on minute-volume output enters another phase of the problem which need not be taken up here.

**METHOD.** Mongrel dogs of medium size were anesthetized with intravenous barbiturates and the chests opened by mid-line incision. Artificial respiration was provided by rhythmic interruption of the communication between a source of compressed oxygen and a tracheal T-tube cannula. After opening the pericardium, the two arms of the myocardiograph were attached to the anterior aspect of the right ventricle by single stitches which did not penetrate entirely through the muscle wall. The rigid arm was attached near the base of the right ventricle at a point clearly below the level of the A-V valves; the movable lever arm was attached at a point well above the apex and at about the position of the intraventricular septum. The diaphragm was removed as a disturbing element by drawing it downward with ligatures anchored low in the abdominal wall. The myocardiograph was of the type first described by Roy and Adami (17) and usually known as the Cusbnny myocardiograph because of his extensive publications based on its use (18-22). The movable lever arm, when connected by thread to the writing lever, required a dead weight pull of about 10 grams to give a typical excursion of one to one and one-half inches. Spring tensions were inserted into this system by means of a coiled wire spring suspended from the upper collar of two movable collars both of which could be fixed with set screws at any de-

sired point along the vertical support rod of the myocardiograph. A plastic block formed the common link to which was attached the lever thread and a thread from the spring. This block was immediately below the side arm of the lower collar and this lower collar was adjusted so that the block nearly impinged on the collar side arm when the heart was at the extreme point of diastolic relaxation. Raising the higher collar now had the effect of adding a spring tension to the regular load on the levers and the block impinging on the lower collar prevented the tension from pulling the heart levers farther apart than the normal point of diastolic relaxation. This arrangement is illustrated in figure 1.

A satisfactory spring was made of 25 gauge B. and S. spring bronze wire wound to give a coil of about 3.5 mm. diameter and 35 mm. length. When stretched, each mm. increase in length corresponded to an increased tension of about 4 grams measured as a dead weight pull. The relation of spring length and tension (in grams) was directly determined and

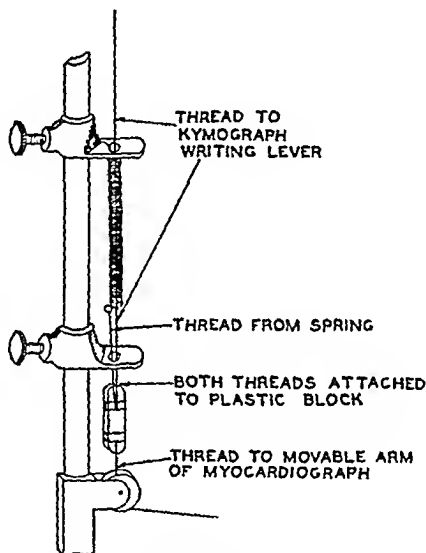


FIG. 1. ARRANGEMENT FOR INTRODUCING VARIABLE SPRING TENSIONS INTO LEVER SYSTEM

plotted over the full range up to a tension of 170 grams. This calibration was checked several times and no important change was noted in the spring characteristics during the course of these experiments. Spring length was determined during the calibrations and the experiments by caliper measurement of the distance between two fixed points on the collars. The isometric systolic tension during the control periods ranged from 20 to 90 grams. This varied according to the size of the heart, the distance between the two points of attachment, the direction of the axis between the two points and the degree of any encroachment over the septum or left ventricle when attaching the lever arm.

Blood pressure was recorded by a mercury manometer connected to one of the common carotid arteries and drugs were infused into one of the external jugular veins. In every experiment, the vago-sympathetic trunk in the carotid sheath was sectioned bilaterally.

**EXPERIMENTAL CONSIDERATIONS.** Experimental variables which might detract from the reliability of the method depend on oxygenation, anesthesia, rotation of the heart axis, irritation due to levers, and spontaneous changes. These are discussed in that order.

The use of compressed oxygen for artificial respiration appeared to be distinctly superior to the use of ordinary air as judged by survival of the hearts and by their relative freedom from irregularities of rhythm. With digitalis overdoses, for instance, the end stages seemed to be deferred and to be less associated with spectacular fibrillary effects than is usually the case with air respiration. During control intervals, it was noted that even when oxygenation appeared to be adequate, an increase of oxygen pressure would at times measurably increase the isometric systolic tension. In so far as possible, oxygen pressure was kept constant, particularly during periods directly before and after drug administration. In the earlier experiments, the oxygen was taken directly from large commercial cylinders equipped with the usual diaphragm regulator. Since this necessitated occasional adjustment of the regulator, the oxygen in later experiments was taken from a floating tank which delivered oxygen to the motor driven interrupter at a constant pressure adjusted within the range of 15 to 20 cm. of water. The respiratory rate was approximately 20 per minute.

The animals were routinely anesthetized with intravenous injections of freshly prepared pentobarbital (about 30 mgm. per kgm.), its proprietary form, veterinary "Nembutal" (about 0.44 cc. per kgm.) or sodium barbital (about 225 mgm. per kgm.). Occasionally, with pentobarbital, there was some lightening of anesthesia in prolonged experiments and supplementary injections had to be given. This occurrence was infrequent and was recognized as affecting any comparisons of drug before and after the supplementary injections. Barbital anesthesia was used in most of the prolonged experiments and, when used, the experiments were not started until 2 to 3 hours after the initial injection because of slow induction; in some such cases, fractional doses of pentobarbital were added before the operation.

Rotation of the heart about its axis was found to affect the determinations. This was possibly due to constriction of the great vessels and possibly to changes in the line of forces within the heart syncytium. This variable was effectively minimized by giving some slight suspension to the heart apex when attaching the levers and by anchoring the horizontal support rod of the myocardiograph by means of heavy cords running transversely above the thorax.

The presence of the levers pulling at two points on the heart surface might naturally be expected to disturb the rhythm of contractions. This however, in the dog heart with an unloaded myocardiograph has been shown to be a negligible factor in the extensive studies of Cushny, Jackson (23) and others. The addition of enough artificial load to bring the levers to a stand-still is recognizably a more extreme type of disturbance. In practice, however, we have found that the loading performance did not obviously interfere with rhythm except on special occasions as when the heart was near failure. Although the length of time for tension determinations was usually kept at a minimum, the heart was shown to be reasonably resistant even to prolonged periods of loading. In one extreme case, the tension was continuously maintained at a point near extinction of the writing stroke through the entire period of control and digitalization which reached a maximum about 40 minutes after infusion was begun. In this experiment there was no unusual frequency of ectopic beats or other obvious disturbances of rhythm. It is probable that the use of whole oxygen atmospheres in the respiratory system was an important factor in minimizing irritability phenomena from this mechanical interference.

With regard to the representative character of this section of the myocardium, it may be pointed out that Cushny observed no important difference between the tracings from either side of the heart and that he preferred to use the anterior aspect of the right ventricle.

Very occasionally, the heart showed a sudden substantial increase of its isometric systolic tension and did so without any evident change in conditions. Spontaneous changes of this sort are unavoidable and their likelihood must be considered in any evaluation of heart responses. The incidence of such changes, however, is low enough not to influence seriously the reliability of effects seen after the injection of quick-acting drugs; with drugs for which prolonged effects are considered, the same degree of reliability is reached only if there is a greater number of experiments. Ordinarily a series of injections was not started until



there was a preceding control period of 20 to 60 minutes with little change in the level of the isometric tension. In one experiment conducted solely to determine duration of regularity, the isometric tension smoothly decreased from 55 to 30 grams over a period of 5 hours, the last 80 minutes of which was without recognizable change; this period was terminated by increasing the oxygen pressure which changed the level to a higher plateau. This control experiment was probably typical of the regularity to be expected when oxygen pressure was controlled by the ordinary commercial regulator; the subsequent use of a constant pressure tank eliminated irregularities due solely to oxygen pressure changes. In one control experiment using the constant pressure tank, the isometric systolic tension dropped slowly from 70 to 65 during the first  $1\frac{1}{2}$  hours, then rose gradually to 90 in the next 2 hours and maintained this level during the final  $2\frac{1}{2}$  hours.

**RESULTS.** Most of the results are included in tables 1 and 2. Table 1 presents the responses obtained with the first injection in each of a number of experiments. Table 2 presents composite results of the subsequent injections in the same series of experiments. In the tables and in the following discussion, the isometric systolic tension is referred to as I.S.T. and the changes expressed in percentages of that in the immediately preceding control period. If, for instance, the I.S.T. in the control period is 40 grams and that after drug administration is 60 grams, the result is expressed as +50%. With the same control, an I.S.T. of 120 grams after drug administration is expressed as +200% and an I.S.T. of 30 grams as -25%. It may be pointed out here that if the I.S.T. during the control period is abnormally low due to cardiac depression, the percentage increase produced by a stimulant drug is likely to be greater than if the heart was at a better functional level.

*All doses are expressed as doses per kgm. of body weight.* The effect of most of these drugs on rate, blood pressure and stroke amplitude is well known and, in the following, the changes in I.S.T. represent the primary interest.

*Digitalis* consistently produced a substantial increase in the I.S.T. which developed relatively slowly and was well sustained. In the case of 1 unit doses, the I.S.T. remained above the control level until shortly before terminal fibrillation. The time for development of peak effects is given in table 1 as time after the beginning of administration, which, as an average, occupied about 22 minutes. Blood pressure, in some cases, was raised as the I.S.T. increased; there was no consistent parallelism, however, and at times the blood pressure fell. Similarly there were distinct divergencies in the stroke amplitude and the I.S.T. despite a parallel course in some cases. When the cardiotonic action of digitalis was well developed, there was a frequent tendency toward rhythmic or phasic variations in the force of the contractions and this was most clearly seen when the spring loading was set at a point near extinction of stroke. When estimating I.S.T. under these conditions, the strong phase was arbitrarily taken as the determining measurement. "Digifolin"-Ciba in ampoules was used in some of the preliminary experiments and in one instance in the final series; no distinction was noted in comparison with the U.S.P. Tincture diluted 1 to 5 in Ringer-Locke solution and this latter was used in all except the one experiment of the final series.

These results constitute an additional quantitative expression of the increased contractile force produced by digitalization. With more rigid standardization

TABLE 1  
Initial injections

DOSE PER KGM.	CHANGE IN ISOMETRIC SYSTOLIC TENSION	CONTROL I.S.T.	TIME FOR PEAK EFFECTS	TIME FOR RETURN TO OR NEAR CONTROL LEVEL	BLOOD PRESSURE CHANGE
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Digitalis

(Administered in instalments over periods of 12 to 40 min.)

	%	gms.	minutes	minutes	mm. Hg.
0.5 U	+37	77	14	50+	+34
0.5 U	+40	90	33	46	+20
0.5 U	+50	70	30	50+	+18
0.5 U	+65	34	30	60+	-40
0.5 U	+70	30	44	50+	+22
1.0 U	+90	53	45	125 (fibrillated)	-18
1.0 U	+100	57	30	120 (fibrillated)	+60 (fig. 4)
1.0 U	+170	44	30	100 (fibrillated)	+80

Epinephrine

mgm.					
0.002	+175	34	$\frac{1}{2}$	3	+30
0.003	+250	20	$\frac{1}{2}$	5	+100
0.010	+180	65	$\frac{1}{2}$	6	+60
0.010	+280	20	$\frac{1}{2}$	4	+120
0.010	+60	40	1	3	+90

Ephedrine

0.2	+40	53	1	6	+40
1.0	+85	53	2	30+	+54 (fig. 2)
1.0	+190	34	2	30+	+54
1.0	+300	18	2	15+	+124
1.0	+180	20	2 $\frac{1}{2}$	18	+100

EA-83

0.25	+30	79	2	6	+4
1.0	+100	25	1 $\frac{1}{2}$	20	+20
2.0	+130	44	1	8	+24
2.0	+75	70	1 $\frac{1}{2}$	7	+20
2.0	+70	75	1	10	+26
10.0	+55	65	1	60	+56
100.0	+50	77	1	30	+50

EA-83 Subcutaneously

100.0	+65	61	5	200	+12
100.0	+50	77	15	220	+40
100.0	+72	77	9	480	+20
100.0	+125	79	10	60	+70
100.0	+77	80	3	60	+24

Caffeine citrate

10.0	0	57	—	—	-60
20.0	+55	25	2	8	+24
20.0	+70	44	2	—	+50

TABLE 1—*Concluded*

DOSE PER KGM.	CHANGE IN ISOMETRIC SYSTOLIC TENSION	CONTROL I.S.T.	TIME FOR PEAK EFFECTS	TIME FOR RETURN TO OR NEAR CONTROL LEVEL	BLOOD PRESSURE CHANGE
Barium chloride					
mgm.	%	gms.	minutes	minutes	mm. Hg.
2.0	+80	48	3	32+	+30
2.0	+45	48	2	30+	+20
10.0	-90	77	5	—	+150
Calcium chloride					
20.0	+45	94	1½	11	+30
20.0	+55	61	1½	7	+24
Potassium chloride					
20.0	+100	30	½	2	+30
20.0	+100	77	½	2½	+76
Quinidine					
2.0	+15	53	1	2½	-54
2.0	+10	57	3	—	-26
Amyl nitrite (by inhalation)					
—	-13	65	½	3	-50
—	-20	70	½	2	-44 (fig. 3)
—	-15	75	1	6	-30

of conditions the method might serve as a bio-assay of digitalis products and might also be used to determine therapeutic indices as well as time relations among various digitalis-like products.

*Epinephrine* hydrochloride in doses ranging from 0.002 to 0.020 mgm. produced in every case a sharp increase in the I.S.T. which returned quickly to the control level or to a lower level. While stroke amplitude usually increased when the I.S.T. increased, the difference in degree was considerable since the stroke amplitude was substantially less sensitive as an indicator. An increase, for instance, of 30% in stroke amplitude was accompanied by an increase of 200% in the I.S.T. In some experiments, the increase of I.S.T. measurably lasted longer than the effects on blood pressure; in general, however, these responses were in close parallel.

*Ephedrine* hydrochloride in initial doses of 1 mgm. consistently produced a sharp rise in the I.S.T. which reached a peak in about 2 min. and remained substantially above the control level for ½ to ¾ hours or more and was associated with considerable rise in blood pressure. The characteristic tachyphylaxis was well demonstrated by the second administration of 1 mgm. doses; in each of 4 experiments the I.S.T. remained virtually unchanged and the mean blood pressure

increase was 23 mm. Hg. Subsequent administration of 1 or 2 mgm. doses produced a reversal of either the I.S.T. or blood pressure or of both. Total doses of 15 to 30 mgm. in 4 to 8 instalments usually brought the experiment to a terminal

TABLE 2  
Composite results of serial injections  
(Excluding the initial injection)

DOSE	CHANGE IN I.S.T.	RANGE	TIME FOR PEAK EFFECTS	TIME FOR RETURN TO OR NEAR CONTROL LEVEL	B.P. CHANGE	RANGE	NO. OF INJECTIONS
<b>Epinephrine</b> (17 injections in 6 experiments)							
mgm. per kgm. 0.002 to 0.020 (av. 10)	+225	(+60 to +500)	$\frac{1}{2}$	4	mm. Hg. (av.) +80	(+30 to +132)	17
<b>Ephedrine</b> (25 injections in 5 experiments)							
0.2 to 0.4	+17	( 0 to +32)	1	6	+15	(+10 to +24)	4
1.0 to 16	-15	(-70 to +30)	—	—	+1	(-35 to +50)	21
<b>EA-83</b> (37 injections in 9 experiments)							
0.25	+42	—	$1\frac{1}{2}$	6	+2	—	1
2.0	+88	(+20 to +300)	$1\frac{1}{2}$	9+	+16	(+10 to +28)	10
3.0 to 10 (av. 5)	+92	(+15 to +185)	2	11+	+17	( 0 to 64)	14
16-100 (av. 62)	+22	( 0 to 35)	2	13	+7	( -4 to +14)	9
200	-31	(-38 to -25)	—	—	-4	( 0 to -10)	3
<b>Caffeine citrate</b> (10 injections in 3 experiments)							
10	0	—	—	—	-35	(-50 to -20)	2
20	+28	( -8 to +70)	$1\frac{1}{2}$	8	+6	(-50 to +24)	8
<b>Barium chloride</b> (8 injections in 2 experiments)							
2	+30	(+20 to +45)	4	17	+30	(+24 to +40)	6
5	+42	(+35 to +50)	$2\frac{1}{2}$	9	+40	(+30 to +50)	2
<b>Calcium chloride</b> (8 injections in 2 experiments)							
20	+45	(+25 to +60)	1	12	+11	( +4 to +24)	5
40	+115	—	$1\frac{1}{2}$	12	+40	—	1
80	+40	(+35 and +45)	2	4	+32	(+32 and +32)	2

TABLE 2—*Concluded*

DOSE	CHANGE IN I.S.T.	RANGE	TIME FOR PEAK EFFECTS	TIME FOR RETURN TO OR NEAR CONTROL LEVEL	B.P. CHANGE	RANGE	NO. OF INJECTIONS
Potassium chloride (6 injections in 2 experiments)							
<i>mgm. per kgm.</i>	<i>av. %</i>		<i>av. in mins.</i>	<i>av. in mins.</i>	<i>mm. Hg (av.)</i>		
20	+82	(+75 to +90)	$\frac{1}{2}$	2	+20	(+16 to +24)	4
20		fibrillated					
60		fibrillated					
Quinidine (10 injections in 2 experiments)							
2 to 20	-25	(-65 to +8)	—	—	-32	(-50 to -10)	10
Amyl nitrite (By inhalation in 3 experiments)							
—	-27	(-40 to -20)	$\frac{1}{2}$	3 $\frac{1}{2}$	-36	(-46 to -30)	5

stage. In one experiment, however, with smaller doses the I.S.T. and blood pressure responses were moderate but positive and without reversal over the course of 7 injections; the dosage order in this case was 0.2; 0.2; 0.2; 0.2; 0.4; 1.0 and 10 mgm. In this case, the last injection increased the I.S.T. about as much as the first while the blood pressure increase was substantially less (8 mm. Hg. as compared with an initial increase of 40 mm. Hg.).

In 3 or 4 experiments, the I.S.T. was reversed first and was relatively more depressed than the blood pressure, while in one experiment the opposite was true. Dilation of the heart was conspicuous in the later stages of ephedrine effects. The direct cardiac depressant action of ephedrine by large or repeated doses is emphasized by these experiments and corresponds to the effect usually seen with the isolated rabbit heart preparations except in the special, narrow range of dosage which produces stimulation.

*2-Methylamino-6-hydroxy-6-methyl heptane hydrochloride.* This aliphatic amine was recently recognized by Jackson to have special stimulant effects on the heart (24). In the following it is referred to by its laboratory number, EA-83. Characteristically, it produced a consistent increase in the I.S.T. with a moderate rise in blood pressure. In contrast to ephedrine, the initial blood pressure rise was less marked and the I.S.T. effect was capable of several repetitions with only occasional reversals under special conditions. In one experiment, the drug was administered 10 times in succession before failure to produce a clear increase in the I.S.T. and blood pressure; the order of doses here was 2; 2; 2; 2; 2; 4; 8; 16; 32 and 64 mgm.; the mean increase in I.S.T. was 35% (variations from 15% to 100%) while the mean blood pressure rise was 12 mm. Hg. (variations from 6 to

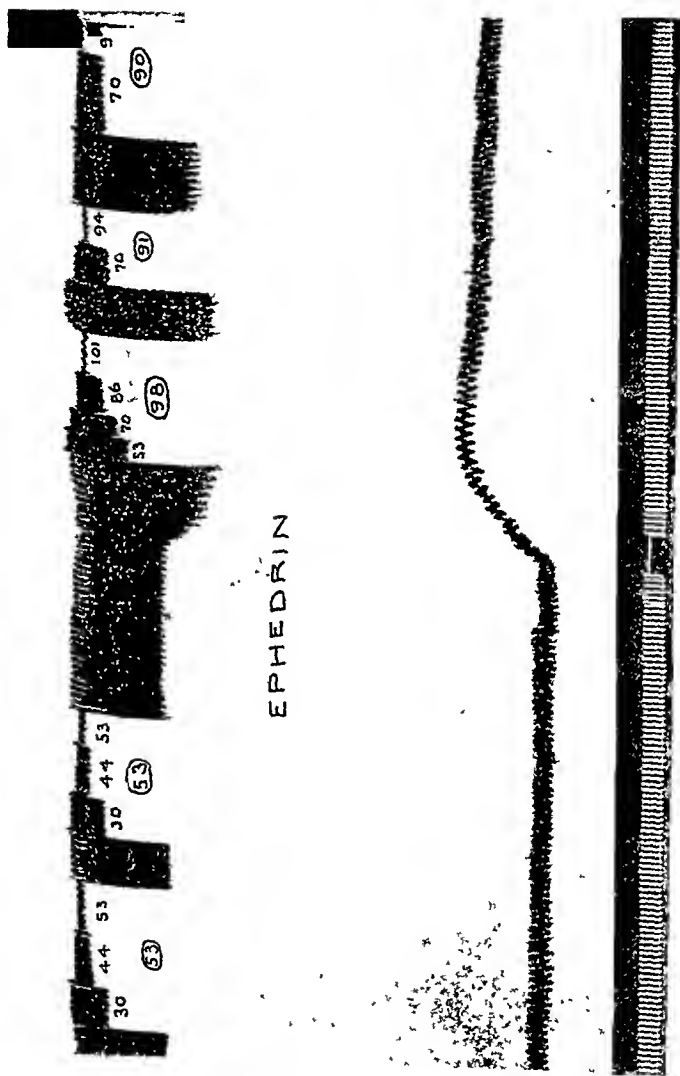


FIG. 2. EPHEDRIN. HCl—1.0 Mm. in Key

Upper tracing—myocardial graph. Downstroke represents systole. Upper row of smaller figures represents load in grams when spring tension is introduced into lever system. Lower row of larger figures (encircled) represents estimated isometric systolic tension. An arbitrarily selected stroke length (ordinarily about 2 mm.) is taken in the control period to represent the point of near extinction. Subsequent stroke lengths are compared to this as a standard. Tracings at several tensions are included here to illustrate the bases for comparisons and estimation.

Lower tracing—blood pressure with Hg manometer. Time marker in 10 second intervals.

20 mm. Hg.). Essentially the same general results were obtained in 5 other experiments in which there were 7 to 8 successive injections averaging a total of 70 mgm. per experiment (variations from 20 to 132 mgm.). The circumstances under which reversal effects occurred were exceptional and involved the use of especially high doses. Following 3 successive injections of 100 mgm., all of which gave positive responses, moderate reversals of I.S.T. and blood pressure were

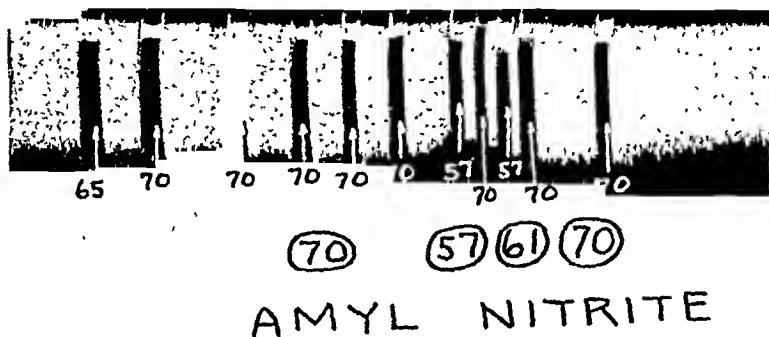


FIG 3 RECORDING IS THE SAME AS IN FIGURE 1 EXCEPT  
THE TIME INTERVALS ARE IN MINUTES

obtained with each of 3 subsequent injections of 200 mgm. each. In another sequence, 2 subcutaneous injections of 100 mgm. each were given, the first of which produced well sustained effects; subsequent injections of 50 mgm. produced a limited and temporary reversal of effect in some instances. In general, it may be said that numerous successive injections of progressively larger doses will be followed by proportionately less marked responses until the heart becomes refractory and, only in exceptional cases of extremely high dosage, is a depressant action obtained on either the I.S.T. or the blood pressure.

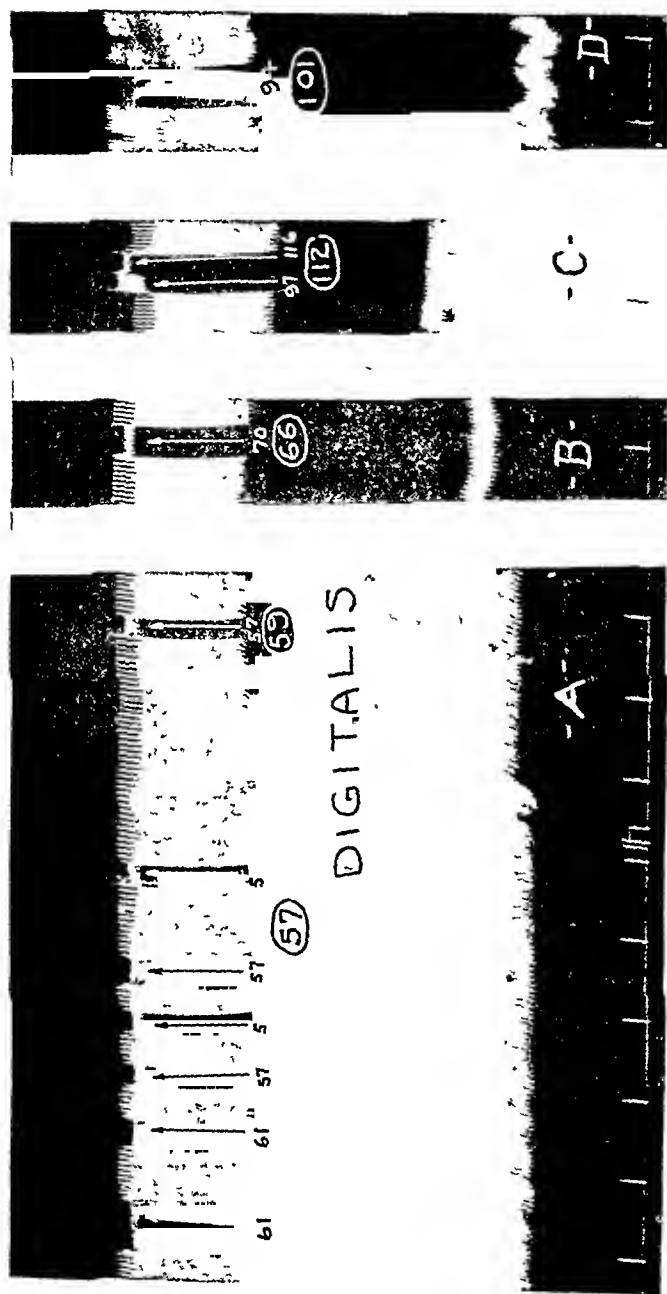


FIG. 1 A Digitalis started. B 16 minutes later C 31 minutes later D 80 minutes later



When there were several instalments before reversal, the total accumulated dose was indicative in the comparison of ephedrine and EA-83. With ephedrine, the total dosages at which reversal of both I.S.T. and blood pressures occurred was 4, 8, 14 and 15 mgm. With EA-83, reversal of either I.S.T. or blood pressure was not obtained with total doses of 20, 20, 48, 45, 92 and 132 mgm.; reversal was obtained with total doses of 362 and 500 mgm.; all of these figures each refer to the total in a series of successive injections in separate experiments. While no rigidly controlled comparisons of myocardial stimulation were made between ephedrine and EA-83 in equivalent initial doses their effects appeared to be generally comparable.

The sequence given above in which 2 subcutaneous injections of 100 mgm. each were followed by 3 intravenous injections of 50 mgm. each was repeated several times for a particular reason. Because of their special character these injections are not included in table 2. The first subcutaneous injection of a relatively large dose (100 mgm.) might be expected to produce a sustained elevation of the I.S.T. This proved to be the case; the mean of the highest levels reached was +78% and the I.S.T. was maintained above the control level for a mean period of 200 minutes. The second subcutaneous injection of 100 mgm. was usually without effect. (These observations demonstrate that prolonged and uniform stimulant action can be obtained by the subcutaneous action of this drug. The period of effects obtained in these experiments, however, cannot be taken as anything more than indicative because of the natural incidence of spontaneous changes in heart performance during prolonged periods.) The subsequent intravenous injections of 50 mgm. produced limited or no responses and an occasional reversal. After these conditions, in which the heart had become incapable of further stimulation with EA-83, administration of digitalis produced distinct and characteristic increases in the I.S.T. This effect was obtained in each of 4 instances in which it was thus administered. The conclusion appears justified that digitalis and this sympathomimetic amine produce their stimulant action through a distinctly different type of cellular attachment. A practical implication also follows in that these experiments suggest that digitalis may be relied on in the usual way after effects of this amine have been manifested.

In contrast, a limited number of trials indicated that EA-83 was without effect after positive responses to ephedrine had been exhausted and likewise ephedrine was without stimulant effect after the heart had become unresponsive to EA-83. Following several injections of barium chloride, EA-83 produced typical stimulation after each of 2 series of injections. EA-83 also produced typical increases in I.S.T. after large doses of quinidine (1 trial), caffeine citrate (2 trials), digitalis (1 trial), and calcium chloride (1 trial); after large doses of octin, EA-83 increased both I.S.T. and blood pressure, but not to the usual degree.

Caffeine citrate was capable of acutely depressing both I.S.T. and blood pressure. More frequently, however, it produced moderate increases in both I.S.T. and blood pressure and subsequent injections, 2 times in one experiment and 5 times in another, were followed by similar responses.

Barium chloride in initial doses of 2 mgm. produced consistent and substantial

increases in both the I.S.T. and blood pressure which were maintained for more than  $\frac{1}{2}$  hour. In one experiment this dose was given 7 times successively with approximately similar but decreasing responses. The period of effects was substantially less with these repeated doses (av. about 16 min.). Ectopic beats were not characteristic under these conditions. Doses of 5 mgm. produced more marked responses. There was good general parallelism of I.S.T. and blood pressure except in the case of one initial dose of 10 mgm. which produced a spectacular rise in blood pressure and marked depression of the I.S.T. Other doses of 10 mgm. given at stages of advanced depression following effects of other drugs produced considerable increase of both I.S.T. and blood pressure in 6 of 7 trials, with a period of rhythm irregularities during the first stage of the effect. The stimulant effects observed here are in contrast to the negative effects reported by Krop (2) using isolated muscle strips and these results emphasize the differences in the two conditions of experimentation. Jackson's (23) observations of amplitude of myocardiograph lever stroke indicate a distinct stimulant action of soluble barium salts.

*Calcium chloride* in doses of 20 mgm. produced prompt and consistent but moderate increases in both I.S.T. and blood pressure and these increases were capable of serial repetition. In contrast to the I.S.T. increase, the blood pressure responses were not maintained as long and, on serial injection, decreased more rapidly.

*Potassium chloride* in doses of 20 mgm., produced effects which in several respects resembled those of small doses of epinephrine, the similarity covering consistency, brevity, repetition and parallelism of blood pressure and I.S.T. This particular similarity of epinephrine and potassium responses may be added to the several others described by Camp and Higgins (25). Terminal fibrillation followed once a dose of 20 mgm. and once a dose of 60 mgm. (This feature of potassium injections has been fully described by Stewart and Smith (26).) The typical increase in contractile force was also obtained 3 times successively following a series of calcium chloride injections.

*Quinidine sulfate* in doses of 2, 2, 5, 5, 10 and 20 mgm. successively produced temporary lowering of blood pressure ( $-54$ ,  $-40$ ,  $-50$ ,  $-40$ ,  $-40$ ,  $-40$  mm. Hg.) with a residue of depression in most cases which brought the blood pressure progressively to a terminal stage. The corresponding I.S.T. effects were respectively  $+15$ ,  $+8$ ,  $-15$ ,  $-30$ ,  $-23$ ,  $-66\%$ . This sequence was repeated in another experiment with essentially the same results. The relatively large doses required for serious depression of the I.S.T. might be considered to favor the contention of Levine (27) that clinical deaths due to quinidine are the result of respiratory depression rather than cardiac depression.

When a sharp fall in blood pressure occurs as was the case here with quinidine as well as with amyl nitrite, direct, primary changes in contractile force are likely to be overbalanced by other more pronounced changes. A lowering of right intraventricular systolic pressure in itself might be expected, through its relief of tension load, to increase the measured I.S.T. Right intraventricular systolic pressure, however, does not follow the same wide fluctuations as does the sys-

temic arterial pressure (28, 29). At the same time, these fluctuations may be related to changes in venous return or intraventricular diastolic pressure and this recognizably is reflected in corresponding changes in contractile force of the heart muscle. When quinidine produces a slight increase in I.S.T., as it does here with small initial doses, suggested but undemonstrated interpretations are that the effect is due to increased diastolic tension or to sympathetic stimulation rather than to any primary increase of myocardial contractile force. The same might be said of the instances in which amyl nitrite produces an increase in I.S.T.

*Amyl nitrite* usually produced a decrease in I.S.T. which closely paralleled the blood pressure fall (fig. 3). The effect in such cases was presumably due to other factors than primary myocardial depression. In one of the 4 experiments with amyl nitrite the results were consistently contrary to those in the other 3 experiments and are not included in the table. In this experiment, amyl nitrite administered 5 times produced decreases in blood pressure ranging from -40 to -60 mm. Hg. with changes in the I.S.T. from +4% to +40%. Subsequent administration of epinephrine in this experiment elicited exaggerated responses and this may have had some bearing on the interpretations.

#### SUMMARY

A method is described by which figures can be obtained representing the isometric systolic tension of a section of myocardium under conditions of an intact circulation. The changes in such tension produced by a series of 10 drugs has been described with respect to dosage, time intervals and associated changes in blood pressure and stroke amplitude.

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## STUDIES ON STREPTOMYCIN

### II. INACTIVATION OF STREPTOMYCIN UPON STANDING IN CERTAIN CULTURE MEDIA AND HUMAN SERUM

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Numerous publications have appeared indicating that streptomycin is quite stable in aqueous solution at ordinary conditions of pH and temperature (1, 2, 3). It is known that sulphydryl compounds (4), ketone reagents (5, 6), and salt (7) inactivate streptomycin. To our knowledge it has not been demonstrated that streptomycin is antagonized by constituents present in bacteriological culture media or human serum. Since much of the *in vitro* work on streptomycin is done in a variety of culture media it was deemed advisable to determine the behavior of streptomycin in such environments.

**EXPERIMENTAL. Media.** To determine the effect of bacteriological culture media on streptomycin, three media were chosen. The first was essentially that proposed by Landy and Dicken (8) consisting of casamino acids, a mixture of vitamins, and inorganic salts (medium #1)<sup>1</sup>, the second was a medium suggested by Waksman and Schatz (2) containing meat extract 0.5%, glucose 1.0%, peptone 0.5%, and sodium chloride 0.5% (Medium #2), and the third was a medium composed of Difco proteose-peptone #3 0.5%, yeast extract 0.3%, meat extract 0.5%, and lactose 0.5% (medium #3). It was felt that these three media covered most of the range of materials used in bacterial growth tests and would give a fairly good indication of the stability of streptomycin in culture menstrua. **Method.** Streptomycin sulfate (Pfizer) in concentrations of 10, 25, 50, 100, 500 and 1000 µg./cc. was added to each of the media mentioned above, and aliquots from each incubated at 4°, 30°, and 37° C. At intervals of four hours and one, two, four, and eight days samples were taken from the tubes and assayed for streptomycin by the paper-disc method of Loo, et al. (9). All samples were assayed in triplicate at two dilution levels. A total of 1620 assays (not including the controls) was run.

**RESULTS IN BACTERIOLOGICAL MEDIA.** Table 1 summarizes a representative sample of the results obtained. Each figure in the table is an average of six determinations and is the percent decrease of recovered streptomycin activity as compared to the initial concentration. It is apparent that the nature of the medium, temperature, time of incubation, and concentration of streptomycin all had a decided effect on the recovered activity of streptomycin under the conditions of

<sup>1</sup> Sodium acetate, asparagine, guanine, xanthine and uracil were omitted from the medium.

The salt concentration of medium #1 was approximately 0.1%. The exact salt concentration of the meat extract, yeast extract, and peptones used in the other two media is unknown. The antagonism of streptomycin activity by salts, previously reported (7), is very slight at salt concentrations of less than 0.5%. The solutions to be assayed in the experiments reported here were diluted prior to assay resulting in salt concentrations far below those which would give antagonism of activity in the bioassay.

the tests. The most marked differences appeared between the media used. Streptomycin introduced in medium #3 lost potency less rapidly and to a smaller degree than in the other two media. In media #1 and #2 there was a loss of as much as 58% in potency of streptomycin within four hours. There was an additional sharp decrease in recoverable streptomycin at one day at all temperatures,

TABLE 1

*Effect of time, temperature, and concentration on inactivation of streptomycin by various culture media as indicated by percentage of decrease of streptomycin compared to concentration originally present*

STREPTOMYCIN μg/cc AT START OF EXPT	TIME	PER CENT DECREASE OF ORIGINAL CONCENTRATION OF STREPTOMYCIN								
		4°C			30°C			37°C.		
		Medium 1*	Medium 2	Medium 3	Medium 1	Medium 2	Medium 3	Medium 1	Medium 2	Medium 3
10	4 hrs.	37	40	0	47	58	10	47	52	0
	1 day	53	53	14	59	47	0	59	35	10
	4 days	63	59	23	62	56	2	68	49	33
	8 days	52	46	21	60	45	37	74	32	2
50	4 hrs.	17	25	0	22	45	0	38	33	6
	1 day	53	48	17	38	39	16	52	38	15
	4 days	52	40	14	56	41	16	69	48	34
	8 days	53	44	3	54	38	21	75	41	20
100	4 hrs.	15	4	0	28	55	0	36	14	10
	1 day	57	25	28	39	35	15	36	28	0
	4 days	48	41	8	52	41	5	50	41	15
	8 days	36	42	9	56	35	7	63	30	0
500	4 hrs.	0	0	0	4	5	0	0	0	0
	1 day	21	24	8	20	29	10	14	22	20
	4 days	25	26	10	30	30	0	36	8	9
	8 days	35	14	13	41	20	3	31	5	10

\* Medium 1 = Medium of Landy and Dicken minus sodium acetate, asparagine, guanine, xanthine and uracil; this medium contains approximately 0.1% salts

Medium 2 = Medium suggested by Waksman and Schatz containing peptone 0.5%, meat extract 0.5%, NaCl 0.5% and glucose 1.0%

Medium 3 = Beef extract 0.5%, yeast extract 0.3%, proteose-peptone #3 0.5%, and lactose 0.5%

Each figure is the average of 6 replicate assays

especially in media #1 and #2. There was a smaller relative loss when streptomycin was present in concentrations exceeding 100 μg/cc. than in more dilute solutions of the antibiotic. Most of the loss in potency occurred during the first twenty-four hours. In general, there was little or no further loss in activity during the next seven days. The loss in recoverable streptomycin assumed the same general time pattern at all temperatures although there was usually a smaller loss of activity at 4°C. than at the higher temperatures. Streptomycin

controls in water at 4°C. showed no loss in activity during the period of the experiments.

**STATISTICAL ANALYSIS.** As indicated by an analysis of variance (10) from the 1620 assays there were significant differences (at 1% but not at 0.1% level of significance) in loss of potency at the various temperatures. Differences in loss of potency between media, at different concentrations of streptomycin, and for varying intervals of time were significant at much less than the 0.1% level.

Because of the unusually large number of replicate assays (six) made for each combination of variables the estimate of the error variance was based on a very large number of degrees of freedom. With the exception of the temperature variable, there is extremely little probability that the differences observed could have arisen from random variation. Even in the case of temperature, which was the least significant of the variables, the chance is only one in one hundred that the differences obtained were due to random sampling.

**RESULTS WITH HUMAN SERUM AND PLASMA.** To test the effect of constituents of serum on streptomycin, the antibiotic was added to human serum to a concentration of 50  $\mu\text{g./cc.}$  and incubated at 37°C. (run in duplicate). At twenty-four hours the solution was assayed for streptomycin. There was a 40% decrease in recoverable streptomycin.

A more elaborate experiment was performed with human plasma. Streptomycin was added to concentrations of 25 and 250  $\mu\text{g./cc.}$  Aliquots of these solutions were incubated at 4°C. and 37°C. Assays for streptomycin were made at four hours and one, two, six and thirteen days. Over the period of the experiment there was a decrease in streptomycin activity of approximately 25% in the more concentrated solutions and 50% in the more dilute solutions. With neither of the two concentrations were there any significant differences in the loss of potency at the two temperatures. As in the case of the bacteriological media, the loss of activity attained a maximum at about one day and, in general, there was little or no further loss. The plasma used contained 1:10,000 merthiolate and sodium citrate as an anticoagulant. Streptomycin was recovered quantitatively at one day from solutions containing merthiolate and sodium citrate in the same concentrations as present in the above plasma.

**DISCUSSION.** The experiments reported here demonstrate that the activity of streptomycin in culture media is affected markedly by the particular medium used, the temperature and time of incubation, and concentration of streptomycin. The activity of streptomycin also was shown to decrease in human plasma.

That the antibiotic is adsorbing onto certain constituents in the environment, in which adsorbed condition it is inactive (at least as determined by the method of assay used), is indicated by the facts that the decrease in potency generally reached a maximum in about twenty-four hours, the relative loss was greater with lower concentrations of streptomycin, and the loss varied with the composition of the environment.

The finding that the activity of streptomycin is greatly affected by constituents of certain bacteriological culture media lends additional support to the suggestion (7) that a standard set of conditions be adopted for such studies in

*vitro* as sensitivity tests. Such a set of conditions should include the following: (a) standard inoculum size, (b) minimal salt concentration, or better, physiological concentration, since the information is usually desired in connection with *in vivo* work, (c) standard temperature, preferably at the optimal temperature for each organism, (d) a standard time interval at which the test is to be read, and (e) use of a medium, the components of which adsorb an insignificant or known amount of the antibiotic. Such a test could be based either on visible growth in serial dilutions of the antibiotic, or on the progress of growth followed turbidimetrically. A decision must be made as to what information is desired from a test of sensitivity. From a clinical standpoint, it is probably desirable to know what concentration will inhibit 100% of a fairly large sample of cells; therefore, it would be necessary to use large inocula and make final readings of the test after at least four days. The adoption of such a standard method would permit comparison of results from different groups of investigators.

#### SUMMARY

The activity of streptomycin was found to decrease while in solution in several bacteriological culture media, human serum, and human plasma. Maximum losses ranged up to 75%. Practically all the loss in activity occurred within the first twenty-four hours. The composition of the environment and the physical factors of time, temperature, and concentrations of streptomycin were found to be significant variables in this loss of potency. It is postulated that the observed loss in potency is the result of adsorption onto constituents of these environments.

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# TREATMENT OF METHEMOGLOBINEMIA

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Methylene blue and ascorbic acid have been shown to reduce methemoglobinemia in animals and in man (1-8). However, there are relatively few data concerning the indications for instituting treatment at various degrees of methemoglobinemia. Moreover, it has not been demonstrated that methylene blue or ascorbic acid can prove a life saving measure at degrees of methemoglobinemia which, without treatment, would prove fatal. Thus, in discussing the treatment of methemoglobinemia in man (9), Wendel stated "The extent to which methemoglobinemia is tolerated by human beings has not been determined. . . . It would seem wise to give immediate treatment to patients with more than 30 per cent methemoglobin."

In the present paper, a more systematic attempt is made to define the indications for treatment of methemoglobinemia. In the course of recent studies of the effect of methemoglobinemia on various physiological functions in man (10, 11, 12), methemoglobin concentrations up to 45 per cent of the total blood pigment were induced. As an extension of these investigations, higher concentrations were induced in the dog and the following studies were carried out: (a) the determination of the incapacitating and lethal levels of methemoglobinemia; (b) a comparison of the effects of various doses of ascorbic acid, methylene blue, and BAL in reducing methemoglobinemia and, (c) an evaluation of the therapeutic effects of methylene blue at lethal levels of methemoglobinemia. The results of these animal studies are reported in the present paper and are correlated with investigations in man and with previously recorded observations on the treatment of methemoglobinemia.

**METHODS.** The effects of methemoglobinemia and of the therapeutic agents were studied in 47 dogs. Methemoglobinemia was induced by the intravenous injection of a propylene glycol solution of p-aminopropiophenone (PAPP) containing 2 mgm. per cc. The concentration of methemoglobin was determined by a slight modification of the Evelyn and Malloy method (13) and expressed as per cent of the total blood pigment; the details have been described in earlier papers (10, 11). In the therapeutic experiments, aqueous solutions of ascorbic acid and of methylene blue and a propylene glycol solution of BAL (2-3 dimercaptopropanol) were injected intravenously.

**RESULTS.** *Symptoms and lethality of p-aminopropiophenone (PAPP) induced methemoglobinemia.* Twenty dogs were injected intravenously with p-aminopropiophenone in propylene glycol in doses ranging from 2.5 to 15 mg. per kgm.

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(table 1) and were followed until death or return to apparently normal behavior occurred. The rate of development of methemoglobinemia and the maximal level attained depended in general upon the size of the dose. To illustrate, the following concentrations of methemoglobin, expressed as per cent of total blood pigment, had developed in 30 minutes: at 2.5 mgm. PAPP per kgm. (dog 2), 44 per cent; at 5.0 mgm. (dog 7), 58 per cent; at 8.0 mgm. (dog 10) 66 per cent. Maximal concentrations of methemoglobin were usually obtained about 1 hour after injection and remained constant for about another hour. The concentration of methemoglobin then began to decline; at the end of 18 to 24 hours after

TABLE 1

*Lethal levels of p-aminopropiophenone (PAPP) induced methemoglobinemia in dogs*

DOG NO.	DOSE OF PAPP	MAXIMAL LEVEL OF METHEMOGLOBINEMIA		FATE
		mgm. per kgm.	Per cent	
1	2.5		61	Survived, normal at 20 hours
2	2.5		61	Survived, normal at 20 hours
3	2.5		58	Survived, normal at 20 hours
4	2.5		45	Survived, normal at 20 hours
5	2.5		53	Survived, normal at 20 hours
6	5.0		75	Survived, normal at 20 hours
7	5.0		73	Survived, normal at 20 hours
8	5.0		81	Survived, normal at 20 hours
9	6.0		89	Died at 183 minutes
10	8.0		89	Died at 85 minutes
11	8.0		89	Died at 65 minutes
12	10.0		83	Died at 300 minutes
13	10.0		87	Died at 103 minutes
14	10.0		86	Survived; Stuporous 24 hours after methylene blue; Hindleg paralysis; Sacrificed.
15	10.0		88	Died at 100 minutes
16	10.0		89	Died at 67 minutes
17	10.0		92	Died at 80 minutes
18	15.0		90	Died at 27 hours
19	15.0		91	Died at 44 minutes
20	15.0		89	Survived; normal at 24 hours.

injection of PAPP the concentrations were negligible, less than 1 per cent of the total blood pigment.

Twelve dogs were observed more closely for the purpose of correlating the level of methemoglobin with the development of symptoms (table 2). No symptoms were evident at concentrations less than 60 per cent methemoglobin. At ranges from 61 to 70 per cent the predominant symptoms were salivation and ataxia, although vomiting was also noted. At a range of 71 to 80 per cent the most frequently noted symptoms were ataxia and vomiting; 2 animals showed prostration. At concentrations from 81 to 90 per cent, loss of consciousness was the outstanding symptom. With one exception (dog 14), animals which survived appeared normal 24 hours after injection. Dog 14 was stuporous and was unable to move its hind legs; it was sacrificed for pathological study.

Of 8 animals which developed maximal concentrations of methemoglobin ranging up to 81 per cent, all survived and were normal during the subsequent period of observation of several days. In contrast, of 12 animals which received higher doses of PAPP and developed maximal methemoglobin concentrations, ranging from 83 to 92 per cent, only 2 survived. Methemoglobinemia developed very rapidly in these animals; 30 minutes after the injection of PAPP, the concentrations of methemoglobin were 70 to 80 per cent. The maximal concentrations were attained about 1 hour after injection and persisted, in the several instances, for a few minutes to 4 hours before death occurred. Of the 2 surviving dogs, one (dog 14) which had developed a maximal concentration of 86 per cent, was stuporous and showed hind leg paralysis 24 hours after the induction of methemoglobinemia. The other animal (dog 20) which had developed a maximal concentration of 89 per cent, survived and appeared normal at the end of 24 hours. It would therefore seem, on the basis of the present studies, that concentrations of PAPP induced methemoglobinemia, greater than 80 to 85 per cent and persisting for several minutes to several hours are almost uniformly lethal.

TABLE 2  
*Symptomatology of PAPP-induced methemoglobinemia*

RANGE OF METHEMOGLOBIN CONCENTRATION	FREQUENCY OF SYMPTOMS NOTED:				
	Salivation	Ataxis	Vomiting	Prostration	Loss of consciousness
<i>per cent</i>					
61-70	5	5	3	0	0
71-80	1	5	6	2	0
81-90	3	0	0	1	6

*Relative effects of methylene blue, BAL, and ascorbic acid in reducing PAPP-induced methemoglobinemia.* Eighteen dogs were injected intravenously with 2.5 mgm. PAPP per kgm. The concentration of methemoglobin was determined 60 minutes later when, as noted in the earlier experiments, the concentration was maximal. Methylene blue, ascorbic acid or BAL was usually injected at this time or a few minutes later when the concentration of methemoglobin was still maximal and the same as that at 60 minutes. The rate of methemoglobin reduction was expressed as the time in minutes required to reduce the methemoglobin to one half its maximal concentration.

Table 3 shows the comparative rates of reduction of methemoglobin in the absence of any therapeutic agent and in the presence of intravenously administered methylene blue, BAL and ascorbic acid. About four hours were required for the spontaneous reduction of methemoglobin to one half its maximal concentration. At a dose of 1 mgm. per kgm. methylene blue, the same degree of reduction was accomplished in 22 to 42 minutes (dogs 22 to 25). Higher doses of methylene blue appeared to accomplish the reduction somewhat more rapidly (dogs 26 to 29). A comparable degree of reduction was attained by BAL at doses of 50 mgm.

per kgm. Ascorbic acid accomplished some reduction (half time, 100 to 204 minutes) at very large doses, 200 mgm. per kgm.

Of the three agents employed above, BAL reduced methemoglobinemia rapidly only at doses which approached the toxic range (14). Ascorbic acid, even in high

TABLE 3  
*Rate of reduction of PAPP-induced methemoglobinemia in dogs*

DOG NO.	DOSE OF THERAPEUTIC AGENT	MAXIMAL CONCENTRATION METHEMOGLOBIN, PER CENT OF TOTAL PIGMENT	TIME REQUIRED TO REDUCE METHEMOGLOBIN TO ONE-HALF THE MAXIMAL CONCENTRATION
Control			
	<i>mgm. per kgm.</i>		<i>minutes</i>
1	—	61	250
2	—	61	205
3	—	58	250
Methylene Blue			
21	0.2	69	175
22	1.0	46	42
23	1.0	55	22
24	1.0	55	31
25	1.0	52	49
26	5.0	56	21
27	5.0	63	28
28	7.0	70	26
29	10.0	64	9
BAL			
30	4.8	65	163
31	4.9	49	60
32	51.1	62	29
33	51.3	60	30
Ascorbic Acid			
34	135	66	No decrease
35	208	51	100
36	200	70	151
37	200	60	204
38	200	54	140

doses, was only slightly effective. It appeared most suitable, therefore, to test methylene blue for its therapeutic effectiveness at lethal levels of methemoglobinemia.

*Therapeutic effect of methylene blue at lethal levels of methemoglobinemia.* Nine animals (dogs 39 to 47) were injected with 10 or 15 mgm. PAPP per kgm., doses which had previously proven fatal. When these animals developed marked symptoms of poisoning such as loss of consciousness or rapid and irregular respira-

tion, methylene blue (1 mgm. and, in two instances, 1.5 mgm. per kgm.) was injected intravenously. The times of methylene blue administration ranged from 45 to 78 minutes after the injection of PAPP and, as may be seen from table 4, the concentrations of methemoglobin were greater than 84 per cent. Of the 9 animals treated with methylene blue, 8 recovered completely. This high rate of

TABLE 4

*Effect of intravenous injection of 1 mgm. per kgm. methylene blue at lethal degree of PAPP-induced methemoglobinemia*

DOG NO	DOSE OF PAPP	INTERVAL BETWEEN PAPP AND METHYLENE BLUE INJECTIONS	CONCENTRATION OF METHEMOGLOBIN AT TIME OF METHYLENE BLUE INJECTION	CONCENTRATION OF METHEMOGLOBIN 1 HOUR AFTER METHYLENE BLUE	NOTE
	<i>mgm per kgm</i>	<i>minutes</i>	<i>per cent</i>	<i>per cent</i>	
39	10	45	86	93	Survived and normal 20 hrs later
40	10	63	84	85	Survived and normal 20 hrs later
41	10	78	84	73	Survived and normal 20 hrs later
42	10	52	87	71	Survived and normal 20 hrs later
43	10	62	85	67	Survived and normal 20 hrs later
44	15	48	88	75	Survived and normal 20 hrs later
45	15*	54	90	78	Survived and normal 20 hrs later
46	15*	55	92	—	Died 5 min after Methylene blue injection
47	15	48	89	82	Survived

\* 1.5 mgm. methylene blue per kgm. injection

recovery is to be contrasted with the low rate of complete recovery, 1 of 12, at these ranges of methemoglobin concentration when no therapy was instituted (table 1).

The improvement in condition following the injection of methylene blue was remarkable. Immediately after the injection, the respiration increased, and within 10 minutes the animals which had been prostrate or unconscious were able to sit up or walk, even though the gait was uncoordinated. At the end of 3 to 4 hours, the animals usually appeared normal. The sequence of symptoms may be

illustrated in greater detail in the case of dog 47. Sixteen minutes after this animal was injected with 15 mgm. PAPP per kgm., ataxia developed; the concentration of methemoglobin at this time was 66 per cent. Salivation and prostration then became marked and the dog lost consciousness. Forty eight minutes after the administration of PAPP when the animal was still unconscious, and the concentration of methemoglobin was 89 per cent, 1 mgm. methylene blue per kgm. was injected. Six minutes later, the animal regained consciousness and sat up. Although it showed some slight ataxia during the next 3 hours, it was able to walk to its kennel and appeared completely normal the next morning (18 hours later).

It may be noted that, although the injection of methylene blue resulted in the very rapid improvement of the condition of the methemoglobin poisoned animals, this improvement was not accompanied by any sudden marked decrease in the methemoglobin levels. At the end of 1 hour, the most marked decrease was to 79 per cent of the level at the time of the methylene blue injection (dog 43) whereas in one instance (dog 39) the concentration increased to 106 per cent of the level at the time of the methylene blue injection. These slight changes in the methemoglobin concentration are to be contrasted with the very rapid decrease occurring with this dose of methylene blue in dogs with non-lethal degrees of 60 to 70 per cent methemoglobinemia.

DISCUSSION. Darling and Roughton (15) have shown that methemoglobinemia, like carboxyhemoglobinemia, shifts the oxygen dissociation curve of the residual oxyhemoglobin to the left and renders the curve less sigmoid and more hyperbolic. The decrease in hemoglobin available for oxygen transport together with the shift of the dissociation curve constitute a potential handicap to the organism in the unloading of oxygen in the tissues. However, it would appear from the studies by Asmussen and Chiodi (16) of carboxyhemoglobinemia in resting man, and by Clark, Van Loon and Adams (17) of methemoglobinemia in the dog that, for levels of inactive pigment up to about 40 per cent, adjustment in the unloading of oxygen in the tissues is accomplished by calling on the reserve or lower half of the oxygen dissociation curve. At methemoglobin concentrations greater than 40 per cent, oxygen supply to the tissues is maintained not only through this mechanism but also by increases in cardiac rate and output (17).

The question naturally arises as to the levels of methemoglobinemia at which the reserve of the oxygen dissociation curve and the circulatory adjustments, combined, prove inadequate in supplying oxygen to the tissues, as judged by the development of symptoms and the occurrence of death. Vandenbelt, Pfeiffer, Kaiser and Sibert (18) found that oral administration of p-aminoceprophenone and p-aminopropiophenone to dogs resulted in ataxia at 60 per cent methemoglobin, salivation and prostration at 75 per cent and loss of consciousness at 85 per cent. Five out of six dogs which developed maximal concentrations ranging from 82 to 87 per cent recovered, whereas 3 of 3 dogs with maximal concentrations of 94 to 95 per cent died. The lethal level of methemoglobinemia was between 87 and 95 per cent. Lester and Greenberg (19) found less marked symptoms in nitrite-induced methemoglobinemia. By injecting repeated small doses

of sodium nitrite into a cat, they obtained increasing methemoglobin concentrations with no ill effects at 66 per cent, ataxia but no unconsciousness at 82 per cent and 87.5 per cent, and ultimate recovery. In a dog which was similarly injected, depression occurred at concentrations of 70 to 80 per cent, ataxia but no unconsciousness at 80 to 85 per cent and prostration at 85 to 89 per cent; the dog recovered. Our results indicate, in general, a similar development of symptoms. The minimal lethal level of methemoglobinemia is somewhat lower (about 80-85 per cent) than that reported by Vandenbelt et al (18).

The present work as well as that of Vandenbelt et al (18) and of Lester and Greenberg (19) represent the highest degrees of methemoglobinemia which have been produced in animals and appear to indicate that the lethality of the compound is due essentially to the conversion of hemoglobin to inactive methemoglobin. The maximal concentrations produced by other methemoglobin formers are less; e.g. in the rat, *p*-aminophenol does not form more than 35 per cent methemoglobin, and  $\beta$ -phenylhydroxylamine not more than 63-66 per cent (20). Increasing the dose beyond that yielding maximal concentration of methemoglobin results in the death of the animal by mechanisms other than by the anoxia due to formation of methemoglobin.

Concentrations of methemoglobin ranging up to 37 per cent have been produced in man in the course of sulfanilamide therapy (3, 4). Bodansky and co-workers (10, 11) induced concentrations up to 30 per cent by the oral administration of *p*-aminopropiophenone. Except for cyanosis which became evident at about 12 per cent methemoglobin, there were in general no complaints or symptoms in these men. Occasionally, an individual would complain of some fatigue following an ordinarily non-fatiguing task. Bannon and Escher (12) produced methemoglobinemias of 40 to 45 per cent in 3 individuals. No symptoms, except cyanosis, were evident at rest or on a mild ambulatory regime. Degrees of methemoglobinemias ranging from 30 to 45 per cent have been reported in several cases of familial idiopathic methemoglobinemia. Of 5 such cases recently summarized by Barcroft et al (21), 3 showed no symptoms while one had dyspnea and headache, and a second dyspnea in severe exercise. The highest degrees of methemoglobinemia in man are those reported by Faucett and Miller (22) in 2 cases of very young infants who had been on a formula which was made up with nitrate-containing well water; the reduction of nitrate led to the formation of methemoglobin, the concentration of which was 58 and 71 per cent in these two cases. At these degrees of methemoglobinemia, these two infants exhibited intense cyanosis, were listless, showed increased heart and respiratory rates, but were not comatose. There have been other instances of severe cyanosis characterized by semi-stupor or by death (23, 24), but no quantitative methemoglobin determinations were recorded in these instances.

On the basis of the available data, it would appear that methemoglobinemias in man up to 30 per cent produce no symptoms, although it has been shown that in moderately severe effort at this degree of methemoglobinemia, oxygenation of the working muscle is impaired (11). In degrees of methemoglobinemia from 30 to 45 per cent, symptoms are absent or slight. It is possible that in the cases of

familial idiopathic methemoglobinemia reported by Barcroft et al (21), the mildness of the symptoms was due to the compensatory development of polycythemia. Concentrations of methemoglobin up to 45 per cent do not therefore, *per se*, constitute an emergency, although it is possible that this degree of methemoglobinemia may, in conjunction with a direct toxic effect on the circulation or respiration, be dangerous. The lethal degree of methemoglobinemia in man cannot be estimated precisely. The recorded existence of 58 and 71 per cent methemoglobinemias without unconsciousness would seem to indicate, by analogy with the symptoms in animals, that the lethal degree may well be above 70 per cent and approach that reported here for the dog.

The rapid reduction of methemoglobinemias by methylene blue in animals previously observed by a number of investigators has also been noted in the present work. It has been shown that the rate of reduction by methylene blue is much greater than that by ascorbic acid or by BAL and it has been demonstrated that methylene blue can prove a life saving measure at degrees of methemoglobinemia which, without treatment, would prove fatal. It is worthy of note that in the present experiments, although methylene blue accomplished rapid abatement of severe and fatal symptoms at extreme degrees of methemoglobinemia, the concentration of methemoglobin was affected only slightly. The mechanism of the action of methylene blue at these ranges of methemoglobin concentrations deserves further study.

Clinical experiences in the treatment of methemoglobinemia may now be briefly reviewed. Wendel (3) treated 6 cases of human sulfanilamide-induced methemoglobinemia, ranging in concentrations from 18 to 29 per cent of the total blood pigment, by the injection of 5 to 13 cc of a 1 per cent methylene blue solution. This was equivalent to a dose of 1 to 2 mgm. per kgm. Forty to 80 minutes after injection, the methemoglobin concentration was found reduced to 0 to 3 per cent of the total blood pigment. Hartmann, Perley and Barrett (4) treated sulfanilamide induced methemoglobinemias in children by the intravenous injection of 1 to 1.5 mgm. methylene blue per kgm. body weight. The concentrations of methemoglobin which were about 20 to 30 per cent before treatment decreased markedly or disappeared in 30 minutes.

Williams and Challis (23) reported a case of methemoglobinemia induced by the contact with p-bromanilin and p-bromorthosulphanilic acid. The patient was intensely cyanotic and semi-comatose when 100 cc of a 1 per cent methylene blue solution (equivalent to about 15 mgm. methylene blue per kgm.) was injected. In 1 hour the patient was revived and the lips and nails were normal in color. In the two cases reported by Steele and Spink (25), one patient was disoriented and the other semi-stuporous; the intravenous injection of 50 cc of 0.5 per cent methylene blue (about 4 mgm. per kgm.) resulted in dramatic improvement. Within 15 minutes after the injection, the patients sat up and appeared well. In the 3 cases described, no quantitative methemoglobin determinations were made.

Comly (5), Ferrant (26), Faucett and Miller (22) have described treatment of nitrite-induced methemoglobinemia in very young infants, due to the inclusion of



nitrate containing well water in the milk formulae. Treatment was carried out by the intravenous injection of 0.5 to 0.6 cc of a 1 per cent solution of methylene blue or a dose of about 1.5 to 2 mgm. per kgm. The report by Faucett and Miller (22) is very instructive. Two infants showed increased respiratory and heart rates and were lethargic. The methemoglobin concentration was 58 per cent and 71 per cent when 0.5 cc and 0.6 cc respectively of a 1 per cent methylene blue solution (equivalent to 2 mgm. per kgm.) were injected. Thirty minutes later the infants appeared clinically well; the respiratory and heart rates had dropped to normal and the methemoglobin concentration was 4 to 5 per cent of the total blood pigment.

Since the lethal degree of methemoglobinemia in man has not been determined, the data which have just been reviewed do not prove that recovery might not have taken place without the institution of therapy. However, these data do show that doses of methylene blue ranging from 1 to 15 mgm. per kgm. are highly and rapidly effective in reducing methemoglobinemia of moderate or severe degree in man. It is of particular interest that, in the only cases (those of Faucett and Miller (22)) in which the existence of grave degrees of methemoglobinemia were quantitatively recorded, the use of a small dose of methylene blue, 2 mgm. per kgm., proved highly effective in the amelioration of symptoms. Our experimental data demonstrate that a dose of 5 mgm. per kgm. possesses slight, if any, advantages over a dose of 1 mgm. per kgm. in reducing methemoglobinemias of 50 to 60 per cent. A dose of 1 mgm. per kgm. methylene blue is highly effective in saving dogs from a degree of methemoglobinemias which, without treatment, would prove fatal. These findings indicate, in conjunction with the clinical data, that small doses of methylene blue, 1 to 2 mgm. per kgm., should be completely adequate in treating methemoglobinemia in man, no matter how severe it may be. That such a dose is well below the toxic level is evident from the report by Nadler et al (27). They injected intravenously 50 cc of a 1 per cent solution (about 7 mgm. per kgm.) in 18 men and observed the chief symptoms to be a sense of oppression in the chest with some difficulty in breathing, occasional dull pains over the heart, a sense of restlessness and apprehension, and fine fibrillary tremors of the face and extremities. There were also increases in heart and respiratory rates, electrocardiographic changes consisting of a reduction in height or even reversal of the T-wave with a lowering of the R-wave, and methemoglobinemias ranging from 0.4 to 8.3 per cent.

Ascorbic acid has also been used in the treatment of methemoglobinemia. For example, Deeny, Murdock and Rogan (6) treated 2 cases of familial, idiopathic methemoglobinemia with doses which began at 50 mgm. twice daily and were increased to maintenance levels of 150 and 200 mgm. twice daily. The blood methemoglobin concentrations after 10 and 13 months' treatment in the 2 cases were 1.3 gm. and 1.25 gm. per 100 cc respectively. In another case with an initial methemoglobin concentration of 46 per cent, Graybiel, Lilienthal and Riley (7) administered 100 mgm. ascorbic acid and 0.6 gm. sodium bicarbonate thrice daily for 14 days at which time the methemoglobin concentration had been reduced to 11 per cent. The dose of ascorbic acid was then increased to 200 mgm.

three times daily. Carnrick, Polis and Klein (8) recently reported the treatment with ascorbic acid of 2 cases of suddenly induced methemoglobinemia. In one case, in which the etiology was not clear, the concentration of methemoglobin was 3.6 gm. per 100 cc (about 25 per cent of the total blood pigment); 72 hours after the administration of 500 mgm. daily by mouth, the methemoglobin was at negligible levels. In a second case, in which the methemoglobinemia was presumably due to ingestion of nitrite, the methemoglobin concentration before treatment was 3.9 gm. Doses of 500 mgm. and, four hours later of 200 mgm. ascorbic acid, were injected intravenously; two hours later or 6 hours after the beginning of therapy, the methemoglobin concentration was 1.9 gm. per 100 cc or one half of its original value.

It may be seen that, in accordance with our experimental data in animals, large doses of ascorbic acid are necessary to reduce methemoglobinemia in man and that the rate of reduction is very slow, as compared with that by methylene blue. There appears to be no question of the advisability of employing methylene blue in acutely induced methemoglobinemias, especially where the symptoms are grave. However, it seems that in familial, idiopathic methemoglobinemia, where repeated daily administration is necessary, ascorbic acid would be preferable to methylene blue.

#### SUMMARY

1. The minimal lethal level of p-aminopropiophenone induced methemoglobinemia in dogs is about 80 to 85 per cent of the total blood pigment.

2. At very high but non-lethal levels, methemoglobinemia is reduced much more rapidly by methylene blue than by ascorbic acid or by BAL. At an intravenous dose of 1 mgm. per kgm. methylene blue is highly and rapidly effective in saving dogs from death at degrees of methemoglobinemia which would otherwise be fatal.

3. A correlation of the clinical literature with the experimental results obtained in the present study, indicates that the minimal lethal level of methemoglobinemia in man approaches that in the dog and that an intravenous dose of 1 to 2 mgm. methylene blue per kgm. is highly and rapidly effective in counteracting the symptoms of severe methemoglobinemia.

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## THE ACTION OF ALSTONINE

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The recent revival of interest in *Alstonia* alkaloids was aroused by the possibility of their substitution for quinine in the treatment of malaria. Leonard and Elderfield (1) elucidated the structure of alstonine, which they isolated from *Alstonia constricta*, and firmly established the presence of  $\beta$ -carboline in the molecule—making the alkaloid similar to yohimbine. Just as Buttle (2) obtained negative results with alstonine sulfate, and Keogh and Shaw (3) with a mixture of *Alstonia* alkaloids, Maier (1) detected no parasitidal action with alstonine in chicks infected with *Plasmodium gallinaceum*.

With a generous supply of crystalline alstonine hydrochloride from Professor Robert C. Elderfield, Department of Chemistry, Columbia University, New York City, we proceeded to make a detailed pharmacologic study. The sample was easily soluble in water, imparting a blue fluorescence. Experiments were carried out to test the effects of alstonine HCl on experimental malaria, blood pressure, respiration, heart, smooth muscle organs, blood sugar, and urine; and to determine its acute toxicity.

**DUCK MALARIA.** Thirty ducklings, 4 weeks old and weighing approximately 400 g. each, were transfused with the blood of a duckling previously infected with *Plasmodium lophurae*, at the height of its parasitemia. The inoculating dose was 2 billion parasitized erythrocytes per kg. of body weight. On the same day, intravenous medication was started—and continued for a total of 5 days—14 ducklings with alstonine HCl, and 6 with quinine dihydrochloride, all 3 times per day. The dose of alstonine HCl varied from 10 to 100 mg. per kg., and that of quinine 2HCl, from 5 to 10 mg. per kg. Ten infected animals receiving no drugs of any kind served as controls. Counting of parasitized red blood cells was made from a smear of each duckling taken on the fifth and sixth days.

Table 1 shows the results of this experiment. The smallest dose of alstonine HCl which reduced parasitemia to an average of below 50 per cent on the sixth day was 15 mg. per kg., and that of quinine 2HCl, 10 mg. per kg. If these doses are compared, it becomes obvious that alstonine HCl is approximately  $\frac{2}{3}$  as active as quinine 2HCl, weight for weight. On the sixth day, the control group (without medication) had an average of 78 per cent parasitemia. The activity of alstonine in duck malaria with *lophurae* infection is of particular interest, because the results of our predecessors (1-3) in other forms of avian malaria were negative. It should also be observed that alstonine HCl in the dose of 50 or 100 mg. per kg. killed the duckling on the first day, due to its own toxic action. Similarly, 2 ducklings treated with 25 mg. of alstonine HCl per kg. of body weight, 3 times a day, showed no parasites in the blood on the sixth

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day, but died on the following day. This is in contrast with quinine 2HCl; which in a wide range of doses completely eradicates the parasites without causing death to the host animal.

**ACUTE TOXICITY.** The median lethal dose  $\pm$  standard error ( $LD_{50} \pm S.E.$ ) of alstonine HCl was determined by intravenous administration in mice and rats. Calculations were made according to the method of Bliss (4). The  $LD_{50} \pm S.E.$  for alstonine HCl was found to be  $8.8 \pm 0.3$  mg. per kg. in mice, and  $14.4 \pm 0.79$  mg. per kg. in rats. Mice evidently are more susceptible to the toxic effects of alstonine than rats. Table 2 summarizes the data. Quinine 2HCl studied under the same conditions showed an  $LD_{50} \pm S.E.$  of  $119.4 \pm 7.43$  mg. per kg. in mice. There is no question that alstonine is much more toxic than quinine.

TABLE 1  
*Action of alstonine in duck malaria with Plasmodium lophurae*

DRUG	DOSE I.V.	NUMBER OF DUCKLINGS USED	AVERAGE PARASITEMIA
	mg. per kg.		%
Alstonine HCl	10	3	56
	15	3	33
	20	3	33
	25	3	10 (2 died on 7th day)
	50	1	(died on 1st day)
	100	1	(died on 1st day)
Quinine 2HCl	5	2	87
	7.5	2	77
	10	2	37
None		10	78

Upon injection of toxic doses of alstonine, the following manifestations were noted in the animals. After an initial increase in the rate of breathing, respiration became irregular and of the gasping spasmodic type. A short period of restlessness followed the injection. Fine generalized muscular tremors often occurred, with a transient coarse quiver spreading throughout the skin over the whole body. When respiration ceased, the animals appeared paralyzed—especially in their hind legs—and went into a state of total flaccidity. No corneal or tendon reflexes could be elicited. No convulsions were observed in any of the animals, not even when fatal doses were administered. Death obviously resulted from primary respiratory failure, since the heart beat persisted for a considerable period after cessation of respiration. The animals that survived the injection of alstonine HCl recovered completely.

Twelve frogs were injected by the ventral lymph sac with a 1 per cent solution of alstonine HCl, the doses varying from 0.32 to 0.93 mg. per g. Within 10 minutes after administration of alstonine, the frogs became lighter in color and

were depressed, with very sluggish reflexes. In 40 to 60 minutes, they were prostrated. Jumping movements were occasionally observed. The abdominal wall of those frogs receiving the larger doses was markedly congested, showing the irritant action of alstonine. After pithing, the chambers of the heart were found to be engorged, and the heart rate was slowed—18 to 35 beats per minute as compared with 60 to 75 in the controls. Two to one A-V block was noted, and this sometimes gradually progressed to three to one, or more. Before the A-V block developed, the A-V interval was quite prolonged as compared with that in the controls.

**ACTION ON BLOOD PRESSURE AND RESPIRATION.** In dogs, under barbiturate anesthesia, intravenous administration of alstonine HCl in the dose of 2 to 5 mg. per kg. consistently produced a sharp fall in blood pressure without any evident change in heart rate. In addition to the marked hypotensive effects, larger doses

TABLE 2  
*Acute toxicity of alstonine HCl*

ANIMAL	DOSE	NUMBER DIED NUMBER USED	LD <sub>50</sub> ± S.E.
	mg. per kg.		mg. per kg.
Mice	8.0	2/10	8.8 ± 0.3
	9.0	7/10	
	10.0	12/15	
Rats	5.0	0/5	14.4 ± 0.79
	8.0	0/5	
	10.0	0/5	
	12.5	3/10	
	14.0	4/10	
	16.0	6/10	
	18.0	5/5	

produced cardiac irregularities. Several minutes after administration of alstonine, intravenous injection of adrenalin resulted in a much smaller rise in blood pressure as compared with the rise brought about by a similar dose of adrenalin given prior to the administration of alstonine (figure 1). This is confirmatory of White's observation in cats (2). Occasionally, the pressor effect of adrenalin in dogs could not be elicited after alstonine. Thus, alstonine resembles yohimbine, not only chemically but also pharmacologically, in that it possesses adrenolytic action. However, in our experiments on dogs, there was no case in which a definite reversal of the adrenalin effect was obtained after administration of alstonine. The cardiac irregularities that appeared under the influence of alstonine were at times temporarily abolished upon injection of adrenalin, but reappeared soon after the effect of adrenalin wore off.

In pithed dogs, the depressant effect of alstonine on the blood pressure was very slight as compared with that in the anesthetized animal. The usual pressor action of various doses of adrenalin was markedly reduced and, in certain

eases, completely abolished in the pithed dog after administration of alstonine. No reversal of the adrenalin effect was obtained after alstonine in the pithed dog.

Under barbiturate anesthesia, the blood pressure of rats was recorded kymographically in the usual procedure by use of a mercury manometer connected either to the carotid artery or to the abdominal aorta at its bifurcation to the common iliaes. Intravenous injection of alstonine HCl in amounts of 1 to 10 mg. (total dose) brought about a sharp fall in blood pressure, the severity and duration of which was roughly proportional to the dose.

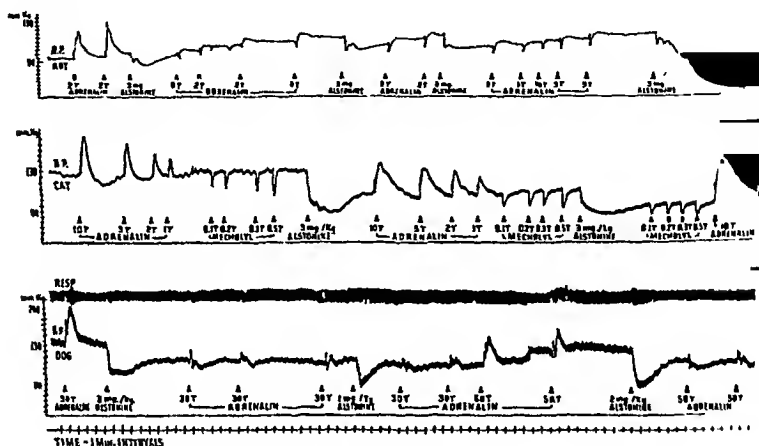


FIG. 1. ACTION OF ALSTONINE ON THE ARTERIAL BLOOD PRESSURE

Top tracing, carotid blood pressure in the rat under pentobarbital sodium anesthesia; middle tracing, carotid blood pressure in the cat under 'Sodium Amytal' (Sodium Iso-amyl Ether) anesthesia; bottom tracing, respiration and carotid blood pressure in the dog under 'Sodium' (Sodium Propyl-methyl-carbonyl Allyl Barbiturate, Lilly) anesthesia. All drugs were given intravenously. After alstonine, the pressor effect of adrenalin was much reduced in the dog and in the cat, and also, usually in the rat. In a few rats reversal of the adrenalin effect was obtained after alstonine; that is, instead of the usual rise of blood pressure, adrenalin, sometimes, produced a fall. This is demonstrated in the rat blood pressure in the top tracing. The hypotensive effect of mecholyl on the cat was not significantly altered by alstonine (middle tracing).

The intravenous administration of various doses of adrenalin, 2 to 15 minutes after the injection of alstonine, was usually followed by a marked reduction in the rise of arterial blood pressure as compared with the rise produced by identical doses of adrenalin given prior to the injection of alstonine. Sometimes, a slight reversal of the adrenalin effect on blood pressure was produced by adrenalin after administration of alstonine. Figure 1 demonstrates the reversal of the adrenalin effect in the rat after alstonine. However, it must be clearly emphasized that in the rat, as in the other species studied, the usual effect of adrenalin after alstonine was a reduction in the magnitude of the pressor effect rather than a constant reversal.

In anesthetized cats, the effects of alstonine on blood pressure were the same as those observed in the dog and in the rat, that is, a marked fall. Similarly, the pressor effects of various doses of adrenalin on the blood pressure were much reduced after administration of alstonine (figure 1), but no reversal of the adrenalin effect on the blood pressure was observed after alstonine HCl. In the cat, Keogh and Shaw (3) obtained a reversal of the effect of adrenalin after injection of *Alstonia* alkaloids, namely, a fall in blood pressure instead of a rise was produced by intravenous administration of adrenalin. The depressor effects of various doses of mecholyl on the arterial blood pressure of the cat were not significantly altered after administration of alstonine (figure 1).

Alstonine HCl caused changes in respiration, as observed in blood pressure experiments. In anesthetized dogs, a dose of 2 to 5 mg. per kg., injected intravenously, resulted in rapid, irregular, and spasmodic respiration for a few minutes, with complete recovery (figure 1)—probably a reflex reaction to the fall of blood pressure. Large doses (10 or more mg. per kg.), however, caused death by respiratory failure, since the heart continued to beat for several minutes. Alstonine had a similar action on respiration of the cat and rat, namely, initial rapid and irregular respiration, followed by gasping, and death from respiratory failure, when toxic doses were administered.

**ACTION ON THE HEART.** In view of the appearance of cardiac irregularities in blood pressure tracings, the effects of alstonine on the electrocardiogram were studied in the dog under barbiturate anesthesia. Control electrocardiograms were taken and followed by intravenous administration of various doses of alstonine. Thereafter, electrocardiograms were taken at repeated intervals. Moderately large doses produced disturbances in initiation (extrasystoles) and in conduction of impulses. Fatal doses brought about an array of disturbances in the electrocardiogram. Figure 2 gives a chronologically detailed picture of the influence of a fatal dose of alstonine HCl, 15 mg. per kg., intravenously administered into a dog. Initially, there was a transient increase in the heart rate which is attributable to the fall in blood pressure as seen in the upper right corner of figure 2. This, however, was soon followed by cardiac failure with severe disturbances in both initiation and conduction of cardiac impulses subsequent to respiratory failure, as seen in figure 2.

An investigation of the effect of alstonine HCl on the isolated heart of the cat, rabbit, and turtle was made. In the rabbit and cat, the coronary arteries were perfused by the Langendorff method. The turtle's heart was perfused by the use of a special glass cannula, introduced through a slit in the left auricle, into which aerated, amphibian Ringer-Locke solution was introduced. The details of the technique were described by one of us previously (5). In all experiments, the drug was dissolved in the perfusing fluid to make concentrations varying from 1:1000 to 1:10,000, and was delivered from a Mariott bottle. A second Mariott bottle containing the non-medicated Ringer-Locke solution was placed at the same level as the first, so that the hydrostatic pressure was the same in both bottles.

Alstonine HCl produced comparatively similar effects on the isolated heart



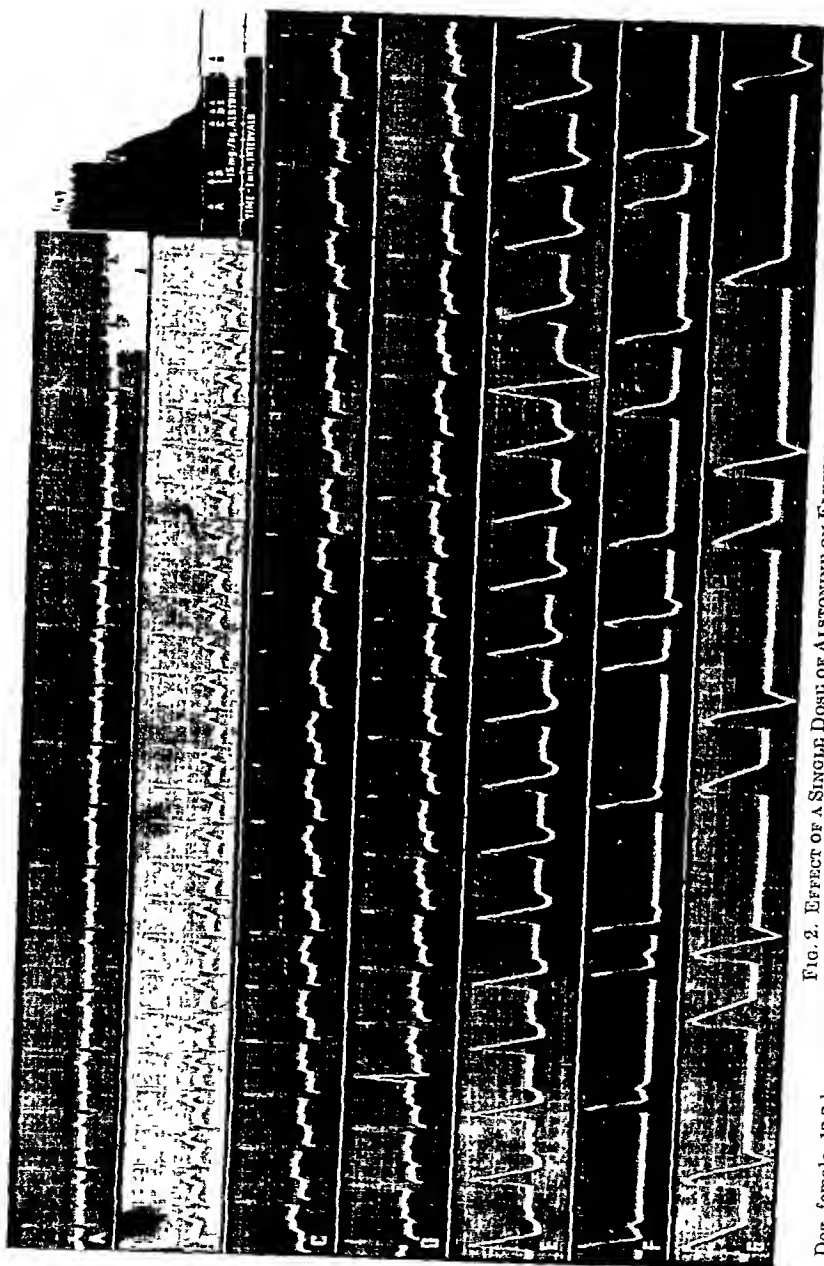


FIG. 2. EFFECT OF A SINGLE DOSE OF ALSTONINE ON ELECTROCARDIOGRAM

Dog, female, 12.2 kg., was anesthetized with 'Sedonal Sodium', injected intravenously. The chronological order of electrocardiograms from Lead II is as follows: A, control; B, immediately after injection of alstonine, 15 mg. per kg.; C, 2 minutes; D, 3 minutes; E, 4 minutes; F, 5 minutes; and G, 6 minutes, after administration of alstonine. The carotid blood pressure, recorded simultaneously, is shown in the upper right corner of the figure.

of the cat, rabbit, and turtle. The severity of effects varied roughly with the concentration of the drug in the perfusing fluid. Initially, the high dilutions produced a transient increase in tone of the heart and a mild reduction in the amplitude of the beats (figures 3 and 4); but upon repeated or continued application, even the high dilutions produced irregularities in rhythm (extrasystoles), ventricular alternations, and A-V block of various degrees; the more concentrated solution led to either ventricular fibrillation or a complete standstill of the heart for a few minutes. Alstonine HCl had a cumulative effect on the heart, for complete recovery could not be obtained even when low concentrations of the drug were replaced by oxygenated Ringer-Locke solution.

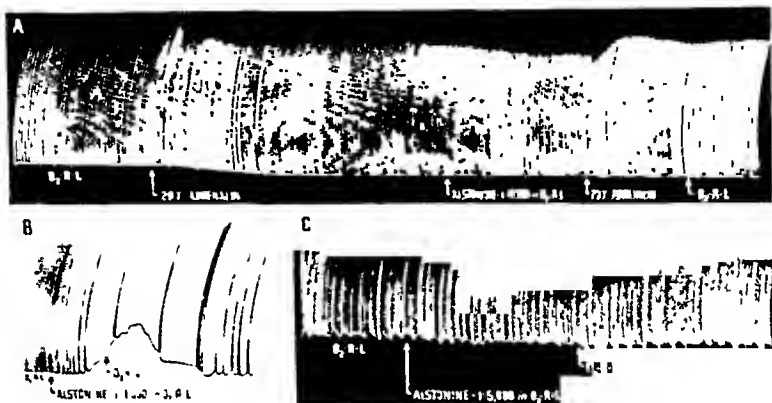


FIG. 3. ACTION OF ALSTONINE ON THE ISOLATED TURTLE HEART

O<sub>2</sub>-R-L designates oxygenated Ringer-Locke solution. A, B, and C are tracings of isolated hearts of 3 turtles. In A, the addition of 20 gamma or  $\mu$ g. of adrenalin to the O<sub>2</sub>-R-L produced a transient increase in the heart rate. The substitution of 1:10,000 solution of alstonine HCl in O<sub>2</sub>-R-L led to slight changes in heart rate and amplitude. Adrenalin had practically the same effect after alstonine as before. In B, a 1:1,000 solution of alstonine HCl in O<sub>2</sub>-R-L led to complete cessation of cardiac activity with a few haphazard beats. In C, alstonine HCl in a 1:5,000 dilution led to reduction in amplitude of the heart beats and irregularity in impulse initiation and conduction.

Administration of adrenalin, before and after alstonine (figure 3), produced practically the same increase in force and rate of the heart.

**SMOOTH MUSCLE ORGANS.** Isolated intestinal strips from various levels of rats, rabbits, and guinea pigs were immersed in oxygenated Tyrode's solution. The action of alstonine HCl on the isolated intestine was indefinite and variable. In dilutions of 1:10,000 to 1:20,000, alstonine produced a progressive reduction in amplitude and rhythmical contractions of the isolated rabbit intestine (figure 5). Occasionally, however, a transient, initial stimulation of intestinal activity was produced by alstonine. Adrenalin produced inhibition of intestinal activity and loss of tone before as well as after alstonine (figure 5). Alstonine HCl often produced inhibition of the normal as well as of the histamine-induced activity in the isolated strip of the guinea pig's intestine.

The effect of alstonine on the isolated intestinal strip from the rat seemed to vary with the tone and state of activity of the strip. When the intestine was in a contracted state, alstonine HCl in dilutions of 1:10,000 to 1:20,000 usually caused relaxation with decrease in amplitude of rhythmical contractions (figure

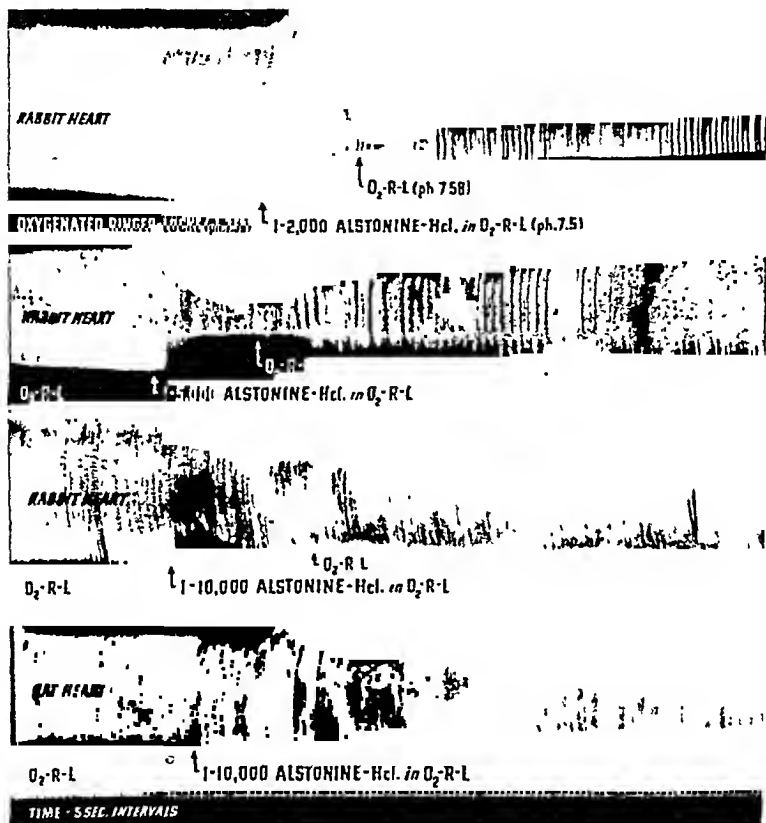


FIG. 4. ACTION OF ALSTONINE ON THE ISOLATED MAMMALIAN HEART (RABBIT AND CAT)

O<sub>2</sub>-R-L designates oxygenated Ringer-Locke solution. Note the rapid deterioration of the heart when a concentration of 1:2,000 to 1:5,000 was used for about 1 minute only. A concentration of 1:10,000 caused progressive deterioration of the heart if applied for a longer period.

5). When the strip was in a state of relaxation, alstonine HCl had no effect on intestinal activity. Similar to the findings in the rabbit and guinea pig, adrenalin caused inhibition of activity and relaxation of tone of the intestine of the rat before as well as after alstonine (figure 5). Alstonine caused prompt relaxation of the intestinal strip that was in a contracted state under the influence

of mecholy; however, it had no effect on the heightened activity of the intestine induced by either potassium or barium chloride.

The effects of alstonine on intestinal activity were studied in 6 trained dogs with exteriorized skin-covered intestinal loops, as described previously (6). Alstonine HCl was given intravenously in doses varying from 1 to 7 mg. per kg. Immediately after injection of the drug, the animals developed generalized, fine muscular twitches with slight tremor. Two of the dogs vomited upon administration of the large doses. Alstonine had either a very slight effect or no influence whatsoever on intestinal activity in trained dogs. The inhibitory effect of adrenalin on intestinal activity was the same before and after administration of alstonine HCl.

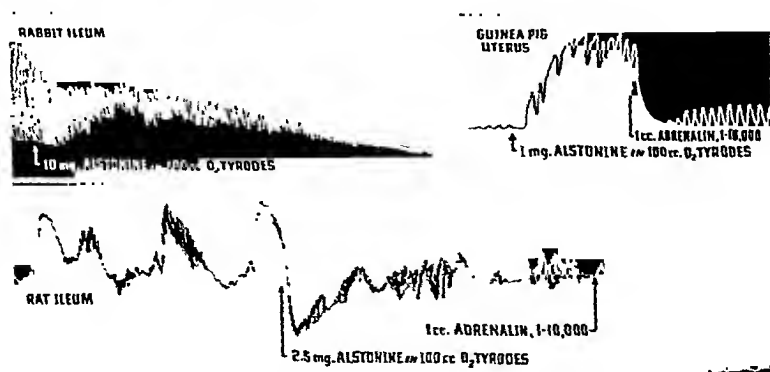


FIG. 5. ACTION OF ALSTONINE ON THE ISOLATED STRIP OF RABBIT'S ILEUM, RAT'S ILEUM, AND OF THE GUINEA PIG'S UTERUS

Note the progressive reduction in the amplitude of the rhythmical contractions of the rabbit's intestine and in the peristaltic action of the rat's ileum. Alstonine stimulated the guinea pig's uterus. Under the influence of alstonine, adrenalin still produced relaxation of the rat's ileum and of the guinea pig's uterus.

Twelve observations were made on isolated uteri of 6 guinea pigs. The addition of alstonine HCl in dilutions of 1:20,000 to 1:100,000 to the isolated uterus in oxygenated Tyrode's solution caused stimulation of the uterus (figure 5), and the height of contraction was roughly proportional to the dose. The administration of adrenalin into the bath in dilutions of 1:50,000,000 consistently caused relaxation of the uterus when it was in a contracted state under the influence of alstonine (figure 5). Evidently, alstonine does not abolish the inhibitory effect of adrenalin on the guinea pig's uterus.

Eight experiments were performed on the isolated uteri of 3 rats. Various doses of alstonine HCl up to a concentration of 1:10,000 produced no noticeable change in the uterus, nor did alstonine alter the relaxation and inhibition of activity produced by adrenalin.

In the pithed dog with bilateral vagotomy, the effects of alstonine HCl on the bronchioles were recorded kymographically by the use of Jackson's method (7).

Intravenous administration of various doses of alstonine HCl produced moderate dilatation of the bronchioles after the administration of ergotoxine, pilocarpine, or histamine (figure 6). The constrictor effect of histamine on the bronchioles was much reduced after administration of alstonine.

In order to determine the effects of alstonine HCl on the eye, a few drops of a 1 per cent solution were instilled into one eye of rabbits, the fellow eye being used as a control. Shortly after instillation, moderate conjunctival injection developed, and a thick yellowish secretion accumulated over the canthi. There was no definite change in the size of the pupil, except in one which developed moderate miosis. No alteration in the light reflex was observed. On the second day and thereafter, the eyes appeared normal, and all conjunctival engorgement and irritation disappeared. After 1 week, the experiments were repeated, and practically the same findings were obtained.

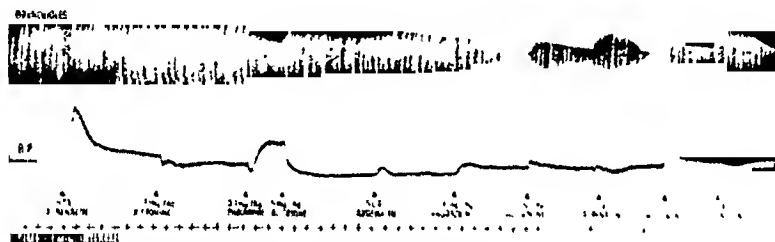


FIG. 6. EFFECT OF ALSTONINE ON THE BRONCHIOLES OF A PITHED DOG

In the upper tracing, a moderate dilator action of alstonine on the bronchioles is obtained after production of bronchiolar constriction by pilocarpine and by ergotoxine. Note that adrenalin causes a more marked bronchiolar dilatation than alstonine.

**ACTION ON BLOOD SUGAR AND URINARY OUTPUT.** The effect of alstonine on blood sugar was investigated in 4 trained dogs. Three of them were each given 5 mg. of alstonine HCl per kg. of body weight, and the fourth, 10 mg. per kg. Repeated determinations of the blood sugar were made for periods varying from 1 minute to 4 hours after administration of alstonine. There was no significant alteration in the blood sugar in any of the dogs.

In dogs under barbiturate anesthesia, the ureters were cannulated in order to study the effects of alstonine on urinary output. Intravenous administration of alstonine HCl in amounts of 3 to 7 mg. per kg. was followed by a transient increase in urine flow, then by a prolonged reduction. It was observed that upon repeated administration of alstonine there was a progressive reduction in the amount of urine secreted until finally a state of anuria occurred. The initial increase in urine formation upon administration of the drug might be due to its irritant effect upon the renal parenchyma. Alstonine was apparently eliminated in large amounts through the kidneys. No matter how small a dose of alstonine HCl was given, the urine of the animal promptly gave off a fluorescence which was most definite under ultraviolet light.

## SUMMARY

1. Alstonine hydrochloride has an antimalarial action against *Plasmodium lophurae* in ducks, being approximately  $\frac{2}{3}$  as active as quinine dihydrochloride. It is, however, much more toxic than quinine as shown in ducklings, mice, and rats.

2. Alstonine HCl lowers blood pressure of anesthetized dogs, cats, and rats, and reduces the response of adrenalin in raising blood pressure (adrenolytic action). Only occasionally does adrenalin reversal occur in rats, but not in cats and dogs.

3. Alstonine HCl in large doses has a deleterious effect on the heart which can be demonstrated electrocardiographically in anesthetized dogs, and by the perfusion of the isolated rabbit's, cat's, and turtle's heart.

4. Fatal doses of alstonine HCl cause primary respiratory failure in anesthetized cats, dogs, and rats.

5. Alstonine HCl frequently inhibits the peristaltic movements of isolated intestines of rats, guinea pigs, and rabbits. It contracts the isolated uterus of the guinea pig, and dilates the bronchioles of pithed dogs previously constricted by pilocarpine or histamine.

6. Alstonine HCl is apparently excreted in urine as judged by the presence of blue fluorescence which it uniformly imparts in aqueous solutions.

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# THE METABOLIC FATE OF ACETANILID AND OTHER ANILINE DERIVATIVES

## II. MAJOR METABOLITES OF ACETANILID APPEARING IN THE BLOOD

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In a previous paper (1) in this series the numerous theories advanced for the metabolism of acetanilid were reviewed and quantitative experimental demonstration was given of the major metabolites of the drug appearing in the urine. The present paper deals with the major metabolites found in blood and plasma. The only previous reports on this subject are the observations of Payne (2) and Young and Wilson (3) of the presence of p-aminophenol in the blood of dogs after administration of large amounts of acetanilid.

The determinations by these investigators were qualitative and no distinction was made between free and conjugated p-aminophenol. Conjugation is strongly suggested because of the excretion in the urine of p-aminophenol as a sulfate or glyeuronate (4) and as the sulfuric acid ester of N-acetyl p-aminophenol after administration of acetanilid to men and animals (5).

The concentration of acetanilid, attained in the blood after its ingestion, as well as the rate of its disappearance, has never been studied. That virtually none is excreted in the urine has been shown (1, 4, 6).

The presence of free aniline in the blood after the administration of acetanilid has never been demonstrated, although its presence has been postulated by Bernheim (7) based on *in vitro* hydrolysis in the presence of animal tissues (8).

From present knowledge, any of the metabolites of acetanilid appearing in the blood might be responsible for the pharmacological and toxicological behavior of acetanilid.

For the present investigation, micro methods were developed for the accurate determination, in blood and plasma, of small amounts of acetanilid and aniline and for the differential determination of free p-aminophenol, N-acetyl p-aminophenol and hydroxy conjugates of N-acetyl p-aminophenol. In view of the striking differences in the metabolism of acetanilid by different species suggested by the literature (1), human subjects were used. Acetanilid was given in amounts corresponding to the ordinary therapeutic doses. The present study includes: (1) serial determinations of the major metabolites of acetanilid in blood and plasma; (2) the renal clearance of each of the metabolites; (3) the penetration of the red cell by each of these metabolites; and (4) the correlation of methemoglobinemia with the occurrence of the various metabolites.

**ANALYTICAL METHODS.** *Aniline and acetanilid.* The specific color reaction occurring when aniline is diazotized and coupled with  $\alpha$ -naphthol, previously described (1), is used

for this determination. The reaction is carried out in a 1:10 protein-free filtrate, deproteinization being accomplished by tungstic acid. One to 5 cc. of filtrate are placed in a 10 cc. glass-stoppered graduated cylinder and 1 cc. of concentrated HCl is added. The volume is made up to 6 cc. with distilled water. One drop of 10 per cent sodium nitrite solution is added and mixed, and 4 drops of a saturated solution of ammonium sulfamate are added. After the evolution of nitrogen has ceased, 2 drops of a 10 per cent alcohol solution of  $\alpha$ -naphthol are added. The mixture is then made alkaline by the addition of 1 cc. of concentrated NaOH and immediately cooled. The volume is made up to 8 cc. with distilled water. Two cc. of *n*-butyl alcohol are added and the color is extracted by shaking vigorously for  $\frac{1}{2}$  minute. Separation of the layers is effected by centrifugation. The supernatant aqueous portion is removed by aspiration and discarded. Concentrated HCl is added to bring the volume to exactly 3 cc. and the mixture is transferred to a micro colorimeter tube and centrifuged briefly, if necessary, to precipitate any sodium chloride. The transmission of the color developed is read in the Evelyn colorimeter, using a filter transmitting at 565  $m\mu$  and set for 100 per cent transmission with a blank determination.

The test-tube holder of the Evelyn colorimeter was adapted to receive the micro colorimeter tube by means of a cylinder inserted into it with light slits measuring 7 mm. in width and 7 mm. in height. The micro colorimeter tube, which fits snugly into this cylinder, is a test tube 15 cm. long and having an internal diameter of 12 mm.

The concentration of aniline, expressed as aniline hydrochloride, in the blood and plasma is calculated from the following equation:

$$\text{Mg. per cent} = \frac{L}{1.016V}$$

in which the constant 1.016 is derived from calibration of the colorimeter with the pigment formed from known amounts of aniline hydrochloride in butyl alcohol-acid mixture.  $L$  is the photometric density of the unknown ( $2\text{-log of the per cent transmission}$ ).  $V$  is the cc. of blood or plasma represented by the volume of filtrate used in the analysis.

The determination of acetanilid is made, as for a conjugated aniline, by preliminary hydrolysis and liberation of free aniline. The conjugated aniline thus determined may be assumed to be acetanilid in the present investigation for two reasons: first, because the compound given in these experiments is acetanilid; and second, the amino group is known to become conjugated in the body only with the acetyl group. One to 5 cc. of protein-free filtrate are placed in a 10 cc. glass-stoppered graduated cylinder. One cc. of concentrated HCl is added, the volume is made up to 6 cc. with distilled water and the mixture is heated for 45 minutes in a boiling water bath. It is then cooled to room temperature and the same procedure is followed as for aniline. The concentration of total aniline, expressed as aniline hydrochloride, in the blood or plasma is calculated from the same equation as for free aniline. The difference between this value and that found for free aniline is taken as acetanilid.

The method described here is specific for aniline, no interference occurring from other derivatives of acetanilid in the concentrations found in body fluids. Analyses of blood and plasma containing added amounts of aniline and acetanilid varying from 0.38 to 0.87 mg. per cent yielded maximum errors of  $+0.03$  and  $-0.05$  mg. per cent. The estimated standard error of the determination was  $\pm 0.02$  mg. per cent. No aniline or acetanilid is lost in the process of analysis.

*Free and total p-aminophenols.* The color reaction occurring when *p*-aminophenol is coupled with  $\alpha$ -naphthol in alkaline solution, previously described (1), is used for determining free and total *p*-aminophenols. The usual deproteinizing agents cannot be employed for obtaining filtrates of blood or plasma because unpredictable losses of *p*-aminophenol occur in the process. The method of deproteinizing described here yields reproducible recoveries of *p*-aminophenol in a 1:10 filtrate. To 18 cc. of 2N HCl in a small flask is added about 0.5 g. zinc dust. Two cc. of blood or plasma are then added and mixed, followed by



2 g. of  $\text{BaSO}_4$ . Two drops of capryl alcohol are added, the mixture is shaken well and filtered. The color reaction of p-aminophenol with  $\alpha$ -naphthol cannot be carried out directly in this filtrate due to the presence of interfering substances and it is therefore necessary to extract the p-aminophenol. For this purpose, 10 cc. of filtrate are transferred to a 100 cc. glass-stoppered cylinder and 0.4 cc. concentrated HCl and about 200 mg. of stannous chloride, to prevent oxidation of the p-aminophenol in the alkaline medium, are added. After mixing, 40 g. of  $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$  are added followed by 24.5 cc. of ethylene dichloride. The mixture is shaken vigorously for 10 minutes in a mechanical shaker. It is then centrifuged and a 20 cc. aliquot of the ethylene dichloride extract is transferred to a 50 cc. glass-stoppered cylinder. Seven cc. of 0.1N HCl are added and the mixture shaken for 5 minutes; the p-aminophenol is extracted as the hydrochloride. Five cc. of the acid extract are transferred to a 10 cc. glass-stoppered graduated cylinder. One cc. of concentrated HCl is added to this, followed by 2 drops of  $\alpha$ -naphthol solution.<sup>1</sup> One cc. of concentrated NaOH is then added and the resulting warm solution is allowed to stand for exactly 3 minutes for color development. It is then cooled, 2.5 g. of KCl are added and 2 cc. of n-butyl alcohol. The mixture is shaken vigorously for  $\frac{1}{2}$  minute for extraction of the color by the butyl alcohol and centrifuged briefly to separate the butyl alcohol. The supernatant aqueous solution is removed by aspiration and discarded. One and one-half g. of anhydrous  $\text{Na}_2\text{SO}_4$  are added to the butyl alcohol extract to remove the water and the mixture is again centrifuged for  $\frac{1}{2}$  minute. The clear butyl alcohol extract is then poured into a micro colorimeter tube and the color density read with a filter transmitting at 635 m $\mu$ . The colorimeter is set at 100 per cent transmission with a blank determination of blood or plasma.

The concentration of free p-aminophenol, expressed as the hydrochloride, is calculated from the equation:

$$\text{Mg. per cent} = 2.70 L$$

in which the value 2.70 was obtained by determinations made on blood and plasma containing added amounts of p-aminophenol hydrochloride. L is the photometric density of the unknown. This method is specific for p-aminophenol, no normal constituent of blood and no other known derivatives of acetanilid producing an interfering color.

In the determination of total p-aminophenol a 1:10 filtrate is prepared as for the determination of free p-aminophenol. Ten cc. of the filtrate are transferred to a 100 cc. glass-stoppered cylinder, 0.4 cc. concentrated HCl and about 200 mg. of stannous chloride are added. Hydrolysis is then carried out by heating for 45 minutes in a boiling water bath. After cooling, the liberated p-aminophenol is determined by the procedure described for free p-aminophenol. The same equation is used for calculating the concentration of total p-aminophenol, expressed as p-aminophenol hydrochloride.

Numerous determinations were made on blood and plasma containing added amounts of free p-aminophenol and N-acetyl p-aminophenol varying from 0.26 to 1.18 mg. per cent. Maximum errors of +0.05 and -0.04 mg. per cent were obtained. The estimated standard error of determination was  $\pm 0.03$  mg. per cent. The amount of p-aminophenol found in the final 0.1N HCl extract used for color development is 78.4 per cent of that which would be expected from the diluting and aliquoting of the original sample. This is due to the mutual solubilities of the ethylene dichloride and the aqueous solutions and mainly to the distribution of the p-aminophenol between these media.

*N-acetyl p-aminophenol.* A 1:10 filtrate is prepared as for the determination of free p-aminophenol. Ten cc. of the filtrate are transferred to a 100 cc. glass-stoppered cylinder, 200 mg. of stannous chloride, 40 g. of  $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$  and 24.5 cc. of ethylene dichloride are added; the N-acetyl p-aminophenol is extracted by 10 minutes of shaking. The mixture

<sup>1</sup> This solution is prepared by allowing 1 cc. of 5 per cent alcohol solution of  $\alpha$ -naphthol to react with 10 mg. of  $\text{K}_2\text{Cr}_2\text{O}_7$  and 1 cc. of 2N HCl for several minutes. Nineteen cc. of 5 per cent alcohol solution of  $\alpha$ -naphthol are then added to this. This preparation of preoxidized  $\alpha$ -naphthol reacts rapidly and completely with p-aminophenol.

is then centrifuged and a 20 cc. aliquot of the ethylene dichloride extract is transferred to a 50 cc. glass-stoppered cylinder. Seven cc. of 0.1N NaOH are added to this and the mixture shaken for 5 minutes thus reextracting the N-acetyl p-aminophenol as the sodium salt. Five cc. of the alkaline extract are transferred to a 10cc. glass-stoppered graduated cylinder. One cc. of concentrated HCl, followed by about 75 mg. of granulated zinc, is added to this, the cylinder stoppered, and the mixture hydrolyzed by heating for 45 minutes in a boiling water bath. After cooling, the color is developed with  $\alpha$ -naphthol and concentrated NaOH, extracted with butyl alcohol, and read in the colorimeter as for free p-aminophenol.

Calculation of the concentration of N-acetyl p-aminophenol, expressed as p-aminophenol hydrochloride, is calculated from the following equation:

$$\text{Mg. per cent} = 2.56 L$$

The value 2.56 was determined from analysis of blood and plasma containing known amounts of N-acetyl p-aminophenol. Since free p-aminophenol is also extracted in the above procedure, a correction must be made for any of it present. For this, the concentration of

free p-aminophenol known to be present multiplied by  $\frac{2.56}{2.70}$  is subtracted from the value

obtained from the above equation. Analyses of blood and plasma containing added amounts of N-acetyl p-aminophenol varying from 0.26 to 1.16 mg. per cent, carried out by this method, yielded maximum errors of +0.03 and -0.03 mg. per cent. The amount of N-acetyl p-aminophenol found in the final 0.1N NaOH extract hydrolyzed and used for color development is 82.7 per cent of that which would be expected from the dilution and aliquoting of the original sample for reasons given for the determination of total p-aminophenol.

The specific determination of N-acetyl p-aminophenol above is possible because the hydroxy conjugates of p-aminophenol with sulfuric and glycuronic acids are not extracted by ethylene dichloride. The latter are not therefore determined directly. However, the sum of the free and N-acetyl p-aminophenol subtracted from the total p-aminophenol equals the hydroxy conjugates of p-aminophenol.

Determinations of the various metabolites in urine, for studying the renal clearances of these substances, were made by methods described in the previous paper (1).

Methemoglobin was determined by the colorimetric method of Evelyn and Malloy (9).

**EXPERIMENTAL RESULTS.** Two normal male subjects in a post-absorptive state were each given 0.975 g. acetanilid orally. Blood was drawn from the antecubital vein at 1, 2, 4 and 6 hours; the amount of methemoglobin and the hematocrit value were determined and both the blood and plasma were analyzed for acetanilid, aniline, free p-aminophenol, total p-aminophenol and N-acetyl p-aminophenol. The concentrations of the hydroxy conjugates of p-aminophenol were calculated. From the concentrations of acetanilid and its metabolites found in the blood and plasma and the hematocrit values, the concentrations in the erythrocytes and the  $\frac{\text{cell}}{\text{plasma}}$  ratios were calculated.

In determining the urinary clearance values, the bladder was emptied  $\frac{1}{2}$  hour before and  $\frac{1}{2}$  hour after the blood sample was taken and the concentrations of acetanilid and its metabolites in the urine determined.

The results obtained are shown in table 1. No free aniline within the limits of the method of analysis used could be detected in the blood or plasma, thus disproving the idea that all or a large portion of the acetanilid is hydrolyzed with the liberation of free aniline. The exclusion of free aniline as a major metabolite does not exclude its occurrence in minute but possibly physiologically active

TABLE 1  
Concentration of various metabolites of acetanilid in blood, plasma and urine after oral administration of 0.975 g. of acetanilid to man

HOURS AFTER ADMINIS- TRATION	BLOOD					PLASMA			CELL RATIO PLASMA		URINE		RENAL CLEARANCE	
	Total p- amino- phenol	N-Acetyl p- amino- phenol	p-Amino- phenol con- jugates	Met- hemog- lobin %	Hema- tocrit. % cells	Acet- anilid	Total p- amino- phenol	N-Acetyl p- amino- phenol	p-Amino- phenol con- jugates	Acet- anilid	N-Acetyl p- amino- phenol	p-Amino- phenol con- jugates	N-Acetyl p- amino- phenol	p-Amino- phenol con- jugates
	mg. %	mg. %	mg. %	mg. %	mg. %	mg. %	mg. %	mg. %	mg. %	mg. %	mg. %	mg. %	mg. %	mg. %
Subject 1														
1	0.69	0.85	0.67	0.18	35	0.75	0.97	0.62	0.35	0.8	1.3	0.0	0.38	8
2	0.49	0.96	0.67	0.29	36	0.43	1.08	0.60	0.48	1.4	1.3	0.0	0.40	9
4	0.16	1.03	0.63	0.40	37	0.13	1.25	0.52	0.73	1.7	1.6	0.0	0.45	9
6	0.12	0.52	0.31	0.21	36	0.11	0.71	0.28	0.43	1.3	1.3	0.0	0.62	13
														cc./min.
														cc./min.
														cc./min.
Subject 2														
1	0.69	1.08	0.87	0.21	37	0.31	1.62	0.86	0.76	0.4	1.0	0.0	0.80	8
2	0.42	1.28	0.92	0.36	38	0.42	1.38	0.67	0.69	1.0	1.9	0.0	2.90	12
4	0.15	1.02	0.69	0.33	34	0.12	1.16	0.57	0.59	1.7	1.6	0.0	2.55	15
6	0.10	0.63	0.37	0.26	38	0.07	0.77	0.32	0.45	2.1	1.4	0.0	2.66	24
														cc./min.
														cc./min.
														cc./min.

The values for acetanilid are expressed as aniline hydrochloride and p-aminophenol compounds as p-aminophenol hydrochloride.

amounts. As will be reported in paper 4 in this series, the use of larger blood samples permits, after the amount of acetanilid given here, the detection of free aniline to the extent of 10 to 20 micrograms per cent.

The concentration of acetanilid in the blood and plasma was highest at the end of the first hour, indicating a rapid absorption of the drug. Thereafter, the concentration fell rapidly. The amounts of acetanilid excreted in the urine were so small as to give renal clearance values of less than 1 cc. per minute.

No free p-aminophenol was found in the blood or plasma. This fact throws doubt on the hypotheses of Heubner (10) and Bernheim (7) that the methemoglobin formed after administration of acetanilid is due to this substance.

The concentration of conjugated p-aminophenols rose during the first hour to a level higher than that of acetanilid. The concentration of p-aminophenols, in contrast to that of acetanilid, was maintained during the first 4 hours in spite of the rapid urinary elimination of p-aminophenols. These findings, together with the fact that acetanilid is eliminated in the urine to only a slight extent, indicate the rapid oxidation of acetanilid to p-aminophenols.

The renal clearance rates for N-acetyl p-aminophenol are low, ranging in the experiments here from 8 to 24 cc. per minute. In contrast, those for the hydroxy conjugates are high, ranging from 93 to 366. As shown previously (1), the major portion of the acetanilid given is accounted for in the urine as the hydroxy conjugates of p-aminophenol.<sup>2</sup>

The metabolism of acetanilid is the oxidation first to N-acetyl p-aminophenol which is slowly eliminated in the urine and second, the conversion of this substance to hydroxy conjugates which are rapidly eliminated in the urine. From the concentration of N-acetyl p-aminophenol and the p-aminophenol hydroxy conjugates developed in the blood in the first hour it would appear that the rate of oxidation of acetanilid to N-acetyl p-aminophenol is more rapid than that of the subsequent conjugation.

The  $\frac{\text{cell}}{\text{plasma}}$  ratios of acetanilid and of N-acetyl p-aminophenol indicate that these substances readily penetrate the erythrocyte and tend to concentrate somewhat within the cell. In contrast, the ratio for the hydroxy conjugates was zero, indicating no penetration of the erythrocyte. The striking difference in this distribution of N-acetyl p-aminophenol and its hydroxy conjugates offers an opportunity to validate the determinations of hydroxy conjugated compounds made here by calculation as the difference between N-acetyl p-aminophenol and total p-aminophenol. For this purpose acetanilid, N-acetyl p-aminophenol and the sulfuric acid ester of N-acetyl p-aminophenol were added to the whole blood and the distribution of these compounds between red cells and plasma was de-

<sup>2</sup> Determinations of the increased excretion of ethereal sulfates and glycuronic acid in the urine after administration of 0.975 g. of acetanilid showed that approximately two-thirds of the p-aminophenol excreted was as the sulfuric acid ester and one-third as the glycuronate. Ethereal sulfate was determined by the method of Tolin (11) and the glycuronic acid by that of Naughan, Evelyn and Browne (12).

terminated. The results obtained are shown in table 2. As may be seen, the  $\frac{\text{cell}}{\text{plasma}}$  ratios of acetanilid and N-acetyl p-aminophenol were similar to those found *in vivo* and that of the sulfuric acid ester was zero, as was also found *in vivo*.

The concentration of methemoglobin in the blood, table 1, reached a maximum of approximately 6 per cent in 2 hours and diminished thereafter at a steady rate. The concentration of acetanilid is highest during the phase of methemoglobin formation but acetanilid *in vitro* does not produce methemoglobin. The

TABLE 2

*Distribution between red cells and plasma of acetanilid, N-acetyl p-aminophenol and the sulfuric acid ester of N-acetyl p-aminophenol added to blood in vitro*

MG. PER CENT IN			HEMATOCRIT	$\frac{\text{CELL}}{\text{PLASMA}}$ RATIO
Blood	Plasma	Cells		
Acetanilid				
0.67	0.59	0.82	33	1.4
0.65	0.56	0.82	33	1.5
0.35	0.30	0.45	33	1.5
0.36	0.31	0.45	33	1.5
N-acetyl p-aminophenol				
0.74	0.57	0.87	37	1.3
1.27	1.21	1.38	37	1.1
1.77	1.53	2.20	36	1.4
N-acetyl p-aminophenol sulfuric acid ester				
1.04	1.62	0.00	34	0.0
0.50	0.77	0.01	36	0.0
0.84	1.78	0.00	39	0.0
0.47	0.83	0.00	39	0.0

presence of a high concentration of p-aminophenols during the period of disappearance of methemoglobin suggests that the pigment does not result from these metabolites. Their possible role in methemoglobin formation, and also that of metabolites appearing in small amounts, will be dealt with in the third and fourth papers in this series.

#### SUMMARY

1. In man, the major metabolites of acetanilid appearing in the blood and plasma are N-acetyl p-aminophenol and its hydroxy conjugates.

2. The renal clearance of acetanilid is insignificant; that of N-acetyl p-aminophenol in two subjects ranged from 8 to 24 cc. per minute; and that of the hydroxy conjugates from 93 to 366 cc. per minute, averaging 174 cc. per minute in one subject and 273 cc. per minute in the other.

3. The major metabolism of acetanilid is its oxidation first to N-acetyl p-aminophenol with slow urinary elimination of this substance and the conversion to hydroxy conjugates with rapid elimination.

4. Free aniline and free p-aminophenol were not found as major metabolites.

5. Acetanilid and N-acetyl p-aminophenol penetrate the erythrocytes and tend somewhat to concentrate within them. The hydroxy conjugates do not penetrate the erythrocytes.

6. The methemoglobinemia occurring after acetanilid does not appear to be due to acetanilid or its major metabolites present in the blood.

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# STUDIES ON THE PHARMACOLOGY OF FLUOROACETATE

## III. EFFECTS ON THE CENTRAL NERVOUS SYSTEMS OF DOGS AND RABBITS

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The toxic actions of the fluoroacetate ion are apparent chiefly in the behavior of the heart and central nervous system (1, 2, 3). Death may result from 1, ventricular fibrillation as the result of progressively more severe cardiac arrhythmias or 2, respiratory depression following repeated convulsive episodes, depending largely upon the species studied. The dose of fluoroacetate required to produce these responses, as well as their relative importance, varies markedly between species (2). Two species, the dog and the guinea pig, are characterized in the intact animal by the complete predominance of the central nervous system effects over the cardiac effects of fluoroacetate, while in a number of species the pattern is reversed.

In a preliminary account from this laboratory of the pharmacological actions of the fluoroacetate ion (1) it was stated that the spike and dome formations characteristic of *petit mal* are present in electroencephalograms obtained from dogs poisoned with fluoroacetate. Although similar disrhythmias can be found in the electroencephalogram of the cat and monkey during fluoroacetate poisoning, considerations presented previously (2, 3) dictated the use of the dog for the majority of the further studies of this phenomenon.

**EXPERIMENTAL.** *A. Behavior of dogs poisoned with fluoroacetate.* Dogs given as little as fifty micrograms of either sodium or methyl fluoroacetate per kilogram of body weight by any route develop convulsions after a period of about two hours during which the animal remains perfectly normal. Central excitation is first manifested by vomiting, barking, snapping and a general behavior pattern suggestive of frightening hallucinations. Aimless running is followed by falling and, at the lower dose ranges, clonic convulsions ensue within three minutes of the first evidence of excitement. In general, periods of clonic seizures which consist of sudden violent jerks of all four limbs at a rate centering about three per second alternate with brief tonic phases or periods of near normalcy during which latter the animal may stand. Smaller doses were not administered. Larger doses (0.5 mgm./kgm.) produce running movements rather than jerking, tonic activity is more marked and periods of normalcy do not appear. Death is invariably the result of respiratory depression under these circumstances.

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Three dogs which had had segments of the spinal cord 2 centimeters in length aseptically removed from the lumbo-dorsal region 10 days prior to the experiments were injected intravenously with 0.1 mgm./kgm. of sodium fluoroacetate. Convulsions of the segments posterior to the transection level developed about one hour after convulsive activity was observed anterior to the level of transection. Death followed fifteen minutes later. In 2 normal dogs intrathecal injection through the  $L_3$ - $L_4$  interspace of 0.5 mgm./kgm. of sodium fluoroacetate produced a typical fluoroacetate convulsion of the hindquarters following a latent period of about thirty minutes. Apprehension was the only change noted attributable to an action on the segments anterior to this region for another thirty minutes but during the next hour the course became similar to that following an intravenous injection of such a dose. It is thus evident that fluoroacetate exerts a broad and powerful action upon the central nervous system. The character of the changes produced in the electroencephalogram is described below.

### B. Electroencephalographic studies.

1. *Method* Suitable leads were placed in the calvarium under local anesthesia (4) The skeletal muscles were insulated from nervous hyperactivity by curare ("Intocostrin") and positive pressure respiration commenced. The cortical potentials were recorded with a four-channel ink-writing electroencephalograph. Monopolar leads against the right ear as reference point were customarily employed, usually with push pull input to the amplifiers. Continuous recordings were made in each experiment.

2. *Results.* a. *Intravenous injection.* In all, 8 dogs were followed in this fashion during the course of poisoning produced by intravenous injections of 0.05 to 1.0 mgm./kgm. of either sodium or methyl fluoroacetate. During all of the experiments there were periods of electrical activity which resembled those obtained during clinical *grand mal* seizures and, similarly, began in a definite area of the cortex under one or two electrodes and subsequently spread to other areas (see figure 1). In general, the area most markedly involved was the parietal region of the cerebral cortex. The frontal and occipital regions were involved slightly or not at all while the cerebellum never evinced any abnormalities. Following episodes of the *grand mal* type, which lasted from a few seconds to many minutes, there were periods of widely variable length during which low voltage slow waves (1-2 per second) dominated the electroencephalogram.

At some time during the course of the poisoning in 6 of the 8 dogs of this series there developed periods in which a spike and dome pattern could be detected. In at least four of these animals there was a definite resemblance of these dysrhythmic waves to those of clinical *petit mal* (5). It was soon apparent that spike and dome waves appeared at various times during the course of poisoning; occasionally shortly before the occurrence of *grand mal* types of activity, but more often after cessation of a bout of such activity.

Although it has been emphasized the fluoroacetate poisoned dogs ordinarily do not manifest the extreme cardiac abnormalities that many other species do (2, 3), under curare and artificial respiration death from respiratory failure can



not occur. Thus, the cardiac effects of fluoroacetate have time to develop and the circulation becomes so poor as to render the cortex electrically inactive. Because of this another type of experiment was performed.

b. *Intracranial injection.* Injections of 41 per cent to 250 per cent of the LD<sub>50</sub> were made through a burr-hole in the temporo-parietal region of the skull. The volume of the physiological saline solutions of fluoroacetate ranged from 0.02

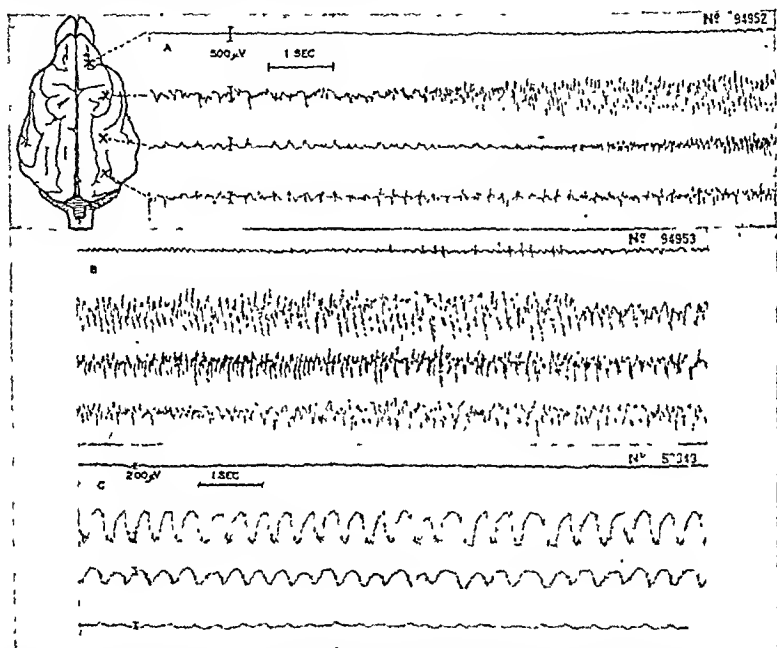


FIG. 1. Dog #2. Sodium fluoroacetate (0.5 mgm./kgm.) was injected intravenously. The onset of a typical *grand mal* electrical seizure 3 hours and 17 minutes later is shown in the continuous tracings "A" and "B". In repeated episodes the area under the second lead near the cruciate fissure always developed such activity in advance of the others, while the most anterior and posterior leads seldom partook. The same localization is seen in "C" during a protracted run of 3 per second high voltage activity with abortive spike formation 4 hours and 22 minutes after the injection.

cc. to 0.40 cc. The procedure of producing a burr-hole and the subsequent injection of similar volumes of physiological saline induced no changes in the electroencephalogram when fluoroacetate was not present. At the end of the experiment the brain, including the medulla, was weighed and the dose of fluoroacetate calculated in micrograms per gram of brain. Cardiac changes were noted only when the largest doses were injected into a lateral ventricle.

Sixteen experiments were performed in this manner using doses ranging from 3.5 to 29.0 micrograms per gram of brain. Both male and female dogs were used ranging in age through 4, 6, 8, and 10 months old puppies to very old animals

and in weight from 5.7 to 25.9 kilograms. No difference in response to the water soluble sodium salt or the highly lipid soluble methyl ester was noted, nor did it appear to be of significance whether the injections were made directly into the cortical substance or into a lateral ventricle. No correlation was observed between any of these factors and the production of the *petit mal* type of electrical activity. Although the frequency of occurrence and the persistence of the *grand mal* type of activity increased generally with the dose of fluoroacetate, no such relationship appeared for the occurrence or persistence of the *petit mal* activity.

Of the sixteen animals examined after intracranial administration of fluoroacetate, 14 at some time manifested varying degrees of spike and dome activity (see figure 2). The two animals which failed to exhibit such activity nevertheless ran typical courses of a *grand mal* character. Those animals which were followed over many hours and which did not develop significant cardiac changes ultimately manifested normal electroencephalograms.

Where the area of poisoning was discrete, such as was the case following injection of small doses into the cortical substance, although both *grand* and *petit mal* activity were present in leads from that region, involvement of other regions spread slowly. With larger doses of fluoroacetate or its injection into a ventricle cortical involvement spread rapidly to include both cerebral hemispheres but never the cerebellum.

It was noted that the amplitude of the abnormal waves seen during fluoroacetate poisoning occasionally reached extremely high levels. A run of 2000 microvolt, 3 per second waves associated with a lower voltage spike is shown in figure 3. Frequently an episode of *grand mal* type of activity showed a definite spike and dome character, seen particularly well when the recording paper was run at a higher speed. Such records are shown in the lower half of figure 3.

When animals were in a state of partial curarization simultaneously with the occurrence of a spike and dome pattern in the electroencephalogram it was noted that the spike was associated with twitching of all four limbs. When more curare was administered the muscular twitch ceased, but the spike and dome pattern continued. This is analogous to the observations of Gibbs, Davies and Lennox that the spike potentials in *petit mal* are usually associated with clonic movements (6).

Barbiturates such as sodium pentothal (5.0 mgm./kgm.) or sodium pentobarbital (20 mgm./kgm.) given intravenously immediately depressed or completely obliterated both the *petit mal* and *grand mal* type of activity, the former type more than the latter. Trimethadione was not adequately evaluated although the impression was gained that it was at least as effective as the barbiturates.

c. *Convulsive action in rabbits.* A number of species has been described (2) which do not develop convulsions following the administration of fluoroacetate. Following the demonstration of the effectiveness of intracerebral injection of fluoroacetate in the dog the procedure was essayed with rabbits, as convenient

representatives of the group of non-convulsing species. Nine animals received doses of sodium fluoroacetate of either 0.10 (2 rabbits) or 0.20 mgm./kgm. (7 rabbits) (40 and 80 per cent of the intravenous  $LD_{50}$ ) through a burr-hole in the skull placed under procaine anesthesia. All developed convulsions similar in

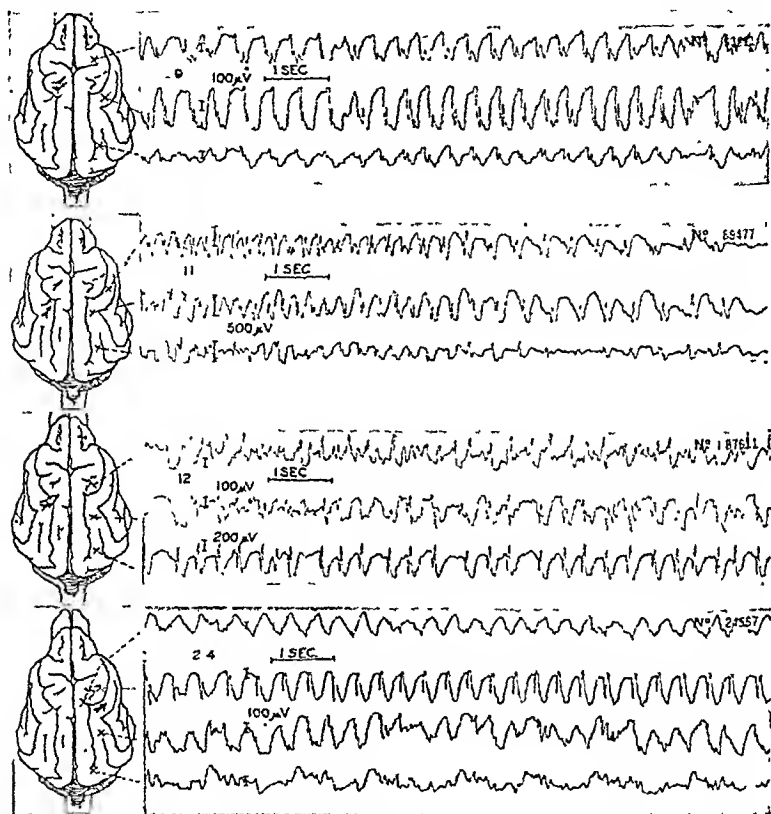


FIG. 2. Four examples are given of spike and dome formation following intracerebral injection of fluoroacetate at the site marked with arrow. Animal 9 received 13.2 micrograms per gram of brain 47 minutes prior to the tracing shown, 11 received 3.5 micrograms per gram of brain 41 minutes previously, 12 received 17.3 micrograms per gram of brain 58 minutes previously and 24, 11.1 micrograms per gram of brain 73 minutes previously. The fourth channel (not shown) recorded the electrocardiograms of animals 9, 11, and 12, which at these times were normal. The second channel of dog 24 shows an unusual inversion of the complex. When inverted this is a well developed, conventional spike and dome formation.

nature to those described for the dog. Eight died, all of respiratory depression. Three control rabbits injected with equivalent volumes of saline and two controls injected with 2.0 mgm./kgm. of sodium acetate remained normal for three days. One rabbit during the present study received 50.0 mgm./kgm. of sodium fluoroacetate intravenously, dying of ventricular fibrillation without any evidence

of excitation thus extending earlier observations (2). It is apparent that rabbit brain, *per se*, is sensitive to fluoroacetate although this is not seen after intravenous injection.

**DISCUSSION.** The observation that convulsions produced by drugs acting on the central nervous system are associated with cortical potentials resembling those obtained during *grand mal* seizures is not unusual. However, the production of electroencephalographic tracings containing the spike and dome pattern considered characteristic of clinical *petit mal* is of interest. As the tracings obtained during fluoroacetate poisoning in the dog appear to differ in some respects from the classical picture presented in the Gibbs' "Atlas of Electroencephalography," it was desirable to compare them with actual records obtained

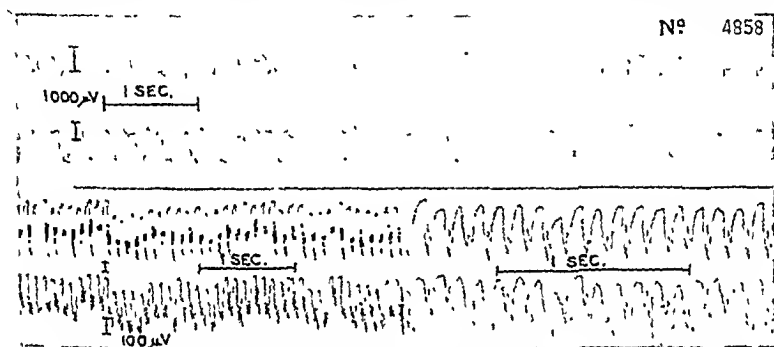


FIG. 3. *Upper tracing.* Dog #2. Obtained 3 hours after the intravenous injection of 0.8 mgm./kgm. of methyl fluoroacetate. Note the very high voltage slow waves despite the administration of dilantin (50 mgm./kgm.) by mouth on each of the 6 preceding days. *Lower tracing.* Dog #26 (15 micrograms per gram of brain). Note the clear-cut spike and dome formations during a *grand mal* type of episode, brought out more clearly by increasing the recording speed.

from patients definitely manifesting clinical *petit mal*. The courtesy of the Electroencephalography Laboratory of the Johns Hopkins Hospital made this possible and it was found that in many instances the experimental tracings bore a closer resemblance to the classical form than did those obtained from actual *petit mal* cases.

There is at present no information which would permit the predication of a relationship between the cerebral dysrhythmias induced by fluoroacetate and those observed in *petit mal*. While such work as has been published to date (7) suggests that fluoroacetate inhibits one or more of the steps in the aerobic breakdown of glucose, these studies do not specifically relate to brain metabolism. Further studies along these lines should be facilitated by the knowledge that rabbit brain, *per se*, is quite sensitive to the actions of fluoroacetate.

#### SUMMARY

1. Spike and dome waves of high voltage occurring at a 3 per second rate have been produced in the EEG of eurarized dogs by the administration of fluoroacetic acid.

2. Six of 8 dogs receiving intravenous injections of 0.05 to 1.0 mgm./kgm. of fluoroacetate (sodium salt or methyl ester) developed cerebral dysrhythmias strongly resembling clinical *grand mal* and *petit mal* seizures.

3. Fourteen of 16 dogs receiving intracranial injections of fluoroacetate (sodium salt or methyl ester) of 41 to 250 per cent of the LD<sub>50</sub> developed similar dysrhythmias.

4. No definite relation was found between the use or dosage of water or lipid soluble fluoroacetates; the sex, age or size of the dog and the occurrence of the *petit mal* type dysrhythmias. The *grand mal* seizures were produced more frequently with larger doses.

5. Rabbits, a species which can not be convulsed by the intravenous administration of fluoroacetates in any dosage, convulse when very small doses (40 and 80 per cent of the intravenous LD<sub>50</sub>) are injected intracranially.

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#### ADDENDUM

While this report was in press a communication, "Convulsive activity induced by fluoroacetate," by A. A. Ward appeared in the *Journal of Neurophysiology*, 26: 105, 1947. Following administration of large intravenous doses of sodium fluoroacetate (2 mgm./kgm.) to cats and dogs electrical activity was enhanced, particularly in subcortical areas.

# THE ANTIHISTAMINE ACTION OF N-(2-PYRIDYL)-N-(2-THENYL)-N',N'-DIMETHYLETHYLENEDIAMINE HYDROCHLORIDE

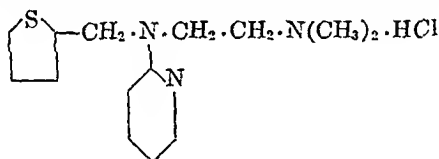
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Since Ungar, Parrot, and Bovet (1), and Bovet and Staub (2) demonstrated the antihistamine effects of certain synthetic compounds more organic chemicals have been shown to have a similar action (3-12). In this country, the therapeutic value of benadryl and pyribenzamine has been established in the treatment of allergic diseases, as recently reviewed (13-14).

In the series of compounds which we investigated, N-(2-pyridyl)-N-(2-thenyl)-N',N'-dimethylethylenediamine hydrochloride (formula shown below) has such a high antihistamine potency that we feel our results merit reporting at this time. For sake of brevity, serial number 01013 will be employed to designate



this compound. Chemically, it has a resemblance to pyribenzamine, differing from it by a thiophene ring in place of benzene. The sample used in the investigation was supplied through the courtesy of Dr. L. P. Kyrides, Monsanto Chemical Company, St. Louis, Missouri. Compound 01013 is a colorless, crystalline powder, melting at 159° to 161°C. (corrected), soluble in water, and bitter to taste.

*Isolated Guinea Pig's Intestines.* When tested on isolated guinea pig's small intestines, usually the ileum, immersed in Tyrode's solution, 01013 in the amount of 0.0025 to 0.01  $\mu\text{g}$ . per cc. of the bath would cause a definite relaxation of a histamine-induced muscle spasm. All the intestinal movements were recorded on a smoked drum. If a constant dose of histamine (calculated as the free base), namely, 0.5  $\mu\text{g}$ . per cc. of the bath, was allowed to act on the intestinal strip for 3 minutes, the extent of relaxation following 01013 at the end of 2 minutes was proportional to the dose of the antihistamine agent—within certain ranges. It was also possible to compare directly the antihistamine action of other compounds, by finding doses which produced equal amounts of relaxation. In order to make the method quantitative, the time of action must be accurately observed—3 minutes with histamine and 2 minutes with the histamine antagonist.

The results of 9 assays on the isolated guinea pig's intestines showed that 01013 was on the average 4.9 times as active as benadryl, but 0.8, as active as pyribenzamine, weight for weight.

The action of 01013 seems specifically antagonistic to histamine. It is not a general spasmolytic agent, since it contracted the isolated virgin rabbit's uterus in concentrations of 1:50,000 to 1:20,000. Nor does it have a definite atropine-like action, because it did not dilate the rabbit's pupil when a 1% solution of 01013 was instilled in the eye, and it did not reduce the submaxillary secretion of the anesthetized dog, induced by intravenous injection of pilocarpine hydrochloride. Although 01013 slightly antagonized the effect of mecholyl on the isolated guinea pig's small intestines, its activity would certainly be less than 1/200 that of atropine sulfate.

*Guinea Pig in Histamine Atmosphere.* The antihistamine action of 01013 was further proved and evaluated in intact guinea pigs. The technique was essentially that of Kallös and Pagel (15). Briefly, the guinea pigs were placed in a desiccator, one at a time, through which an atmosphere containing atomized histamine solution was continuously passed. The concentration of histamine in the form of acid phosphate was 0.187  $\mu$ g. per liter of air, and the rate of flow of histamine vapor was approximately 9 liters per minute. An untreated guinea pig in this atmosphere usually developed restlessness, dyspnea, and convulsions in 1 to 3½ minutes. If 01013 were injected subcutaneously prior to exposure to histamine vapor, the pig could be protected from the toxic action of histamine; the larger the dose of 01013, the longer the protection. If the animal failed to develop convulsions within 5 minutes, it was considered to be protected. By exposing treated guinea pigs to the histamine atmosphere 1 hour after injection of various doses of 01013, subcutaneously, the median protective dose (the amount necessary to protect 50% of the animals) could be determined. For example, in an experiment carried out with the same colony of guinea pigs, the doses of 01013 per kg. of body weight protecting the number of pigs out of the number injected were as follows: 1 mg., 12/12; 0.5 mg., 10/12; 0.275 mg., 8/12; 0.14 mg., 5/12; 0.07 mg., 4/12; and 0.0365 mg., 1/12. The computed median protective dose  $\pm$  standard error ( $PD_{50} \pm S.E.$ ) by the Bliss method (16) was therefore  $0.152 \pm 0.023$  mg. per kg. By the same method, the  $PD_{50} \pm S.E.$  of benadryl was found to be  $1.27 \pm 0.36$  mg. per kg.; and that of pyribenzamine,  $0.059 \pm 0.015$  mg. per kg.

*Action on Blood Pressure.* In anesthetized cats, 01013 reduced the response of blood pressure to histamine, both administered intravenously, although a large dose (10 mg.) of the former alone caused a sharp fall of blood pressure with slow recovery. The antagonism is illustrated in figure 1, where the threshold dose of histamine was raised at least eightfold following the injection of 01013. The inhibition of histamine action on blood pressure was also observed by the oral administration of 01013; a dose of 15 mg. per kg. completely abolished the depressor effects of 0.5  $\mu$ g. of histamine.

The pressor action of epinephrine, on the other hand, appears to be augmented by 01013. In figure 2, it can be noted that a greater rise of blood pressure occurred with the same dose (10  $\mu$ g.) of epinephrine after 01013 in the dose of 1 mg. per kg., also given by vein. In the same experiment, the hypotensive effect of 1  $\mu$ g. of histamine was obviously nullified. The potentiation of action of





epinephrine by benadryl and pyribenzamine has been respectively reported (17-18).

**Toxicity.** The acute toxicity of 01013 was determined in starved albino mice. By intravenous injection, the doses of 01013 per kg. of body weight killing the number of mice out of the total number injected were as follows: 16 mg., 1/10; 18 mg., 8/20; 20 mg., 11/20; and 22.5 mg., 14/20. By mouth, the doses killing the number of mice out of the total number used were as follows: 100 mg., 0/5; 150 mg., 3/10; 200 mg., 6/10; 250 mg., 9/10; and 400 mg., 10/10. The median lethal dose ( $LD_{50} \pm S.E.$ ) in mice by vein is therefore  $19.85 \pm 0.69$ , and by mouth,  $182.2 \pm 12.8$ , mg. per kg. For comparative purposes, benadryl and pyribenzamine were injected intravenously to mice, the  $LD_{50}$  of the former being  $29.70 \pm 1.67$ , and that of the latter,  $18.26 \pm 0.60$ , mg. per kg. It is clear that benadryl is the least toxic of the three.

Ten mice were given 20-mg.-per-kg. doses of 01013, daily, for 4 weeks. Two mice died of mechanical injury. The 8 surviving animals gained weight, and upon sacrifice showed no pathologic lesions, grossly or microscopically. There is little doubt, therefore, that the drug in this dose can be tolerated by mice for a long period of time.

**In Man.** Seven subjects volunteered to take 01013 by mouth in doses varying from 10 to 50 mg. The drug was dispensed in capsules. One of the individuals experienced a "light feeling," and another expressed, "stomach irritated," both with a dose of 30 mg. All other persons, including 4 ingesting 50 mg. each, were entirely free from any form of reaction.

During the fall of 1946, Dr. M. H. Mothersill, of our medical division, administered 01013 to a group of 29 hay fever sufferers—caused by ragweed pollen. A dose of 25 mg. per os, repeated once or twice a day, alleviated coryza, epiphora, and sneezing in the majority of cases. These suggestive results with 01013 warrant a further clinical trial.

#### SUMMARY

1. N-(2-Pyridyl)-N-(2-thenyl)-N',N'-dimethylethylenediamine HCl, designated as 01013, has a potent antihistamine action as proved by its inhibition of histamine effect on the isolated guinea pig's small intestines, the intact guinea pig placed in a histamine atmosphere, and the blood pressure of anesthetized cats.
2. The compound augments the pressor action of epinephrine.
3. Its toxicity in mice has been determined.
4. There is evidence that 01013 given by mouth may be efficacious in the treatment of hay fever.

Grateful acknowledgment is made to Dr. Paul N. Harris and Mr. Robert C. Anderson for their invaluable assistance in connection with the toxicity studies.

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# ANESTHESIA

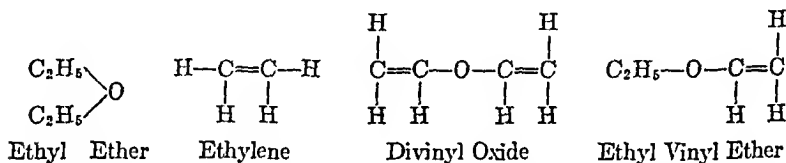
## XXVIII. THE ANESTHETIC ACTION OF ETHYL VINYL ETHER\*

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At the suggestion of Leake (1) divinyl oxide was prepared by Major and Ruigh (2) and studied by the former investigators as an anesthetic agent. The usefulness of the compound for anesthetics of short duration has been established. Divinyl oxide has been referred to frequently as the hybrid molecule between ethyl ether and ethylene. It occurred to us that ethyl vinyl ether represents more completely a cross between the two anesthetic molecules than does divinyl oxide. The relationship of these compounds to ether and ethylene is apparent from the following formulas:



Leake (1) obtained a small sample of ethyl vinyl ether from Prof. S. Fraenkel of Vienna in 1930. In studies on 4 mice, Leake reported the compound to exhibit more potent anesthetic properties than ethyl ether, although he found it to have an oil/water coefficient of  $0.5 \pm 0.1$ . Shostakovskii (3) found the agent to exhibit anesthetic properties in frogs but to be less potent than ether.

It was our opinion that it would be interesting to submit this compound to complete anesthetic studies on several species of animals as we have studied previously cypreth (4) and other hybrid ethers.

Ethyl vinyl ether is a volatile, colorless liquid with an odor which resembles that of divinyl oxide: the boiling point is  $35.8^\circ\text{C}$ . and the specific gravity 0.755 at  $20^\circ\text{C}$ .

*Anesthesia in the monkey.* Two large *Macacus rhesus* monkeys were anesthetized with ethyl vinyl ether using a closed circuit with oxygen. The technique is described in our former studies (5). The induction period was much more rapid than with ethyl ether, indicating a greater potency. Surgical anesthesia was uneventful; breathing was stertorous, deep and regular. Recovery from the anesthesia was prompt; far more rapid than with ethyl ether. The quantities of the agent employed were approximately one-half those used to produce similar anesthetic syndromes with ethyl ether.

*Anesthetic index (dog).* The dogs employed were fed a diet of "Purina Chow"

\* The expense of this investigation was defrayed in part by a grant from the Ohio Chemical & Mfg. Co., New York, N. Y.

one week prior to the experiment and fasted 12 hours immediately before anesthetizing. The procedure was identical with that employed in our cyprome ether studies (6). The number of cubic centimeters of the agent per kilogram required to produce surgical anesthesia was divided into the volume required to produce respiratory arrest. The quotient was designated as the anesthetic index. The results are summarized in table 1.

The data in table 1 indicate that ethyl vinyl ether has a wide margin of safety. The mean difference between the induction dose and the quantity required to produce respiratory failure is 1.1 cc. This value is approximately the same for ethyl ether, n-propyl methyl ether and ethyl vinyl ether. With divinyl ether 0.3 cc./Kg. was required to produce anesthesia, with ethyl vinyl ether 0.56 cc./Kg. and with diethyl ether 1.1 cc./Kg. These data demonstrate the increased anesthetic potency conferred on the molecule by unsaturation.

*Blood pressure studies (dog).* The effect of ethyl vinyl ether on the blood pressure was determined by anesthetizing the animal with ethyl ether. The

TABLE 1

DOG NUMBER	SEX	WEIGHT	INDUCTION	RESPIRATORY FAILURE	ANESTHETIC INDEX
		Kg.	cc./Kg.	cc./Kg.	
1	F	6.0	0.58	1.58	2.72
2	F	5.6	0.63	1.70	2.72
3	M	11.2	0.54	1.92	3.56
4	F	9.0	0.44	1.66	3.77
5	F	6.0	0.50	1.17	2.34
6	F	8.0	0.56	1.38	2.46
7	F	8.0	0.63	1.63	2.59
8	M	7.5	0.60	1.93	3.22
9	F	5.5	0.55	2.09	3.80
10	F	7.5	0.53	1.47	2.77
Mean.....			0.56	1.66	3.00

blood pressure was determined in the usual manner by cannulating the carotid artery. The ethyl ether was removed and ethyl vinyl ether used as a substitute anesthetic agent. The respiratory tracings were made by means of a chest rubber tambour. The anesthesia was deepened to the point of respiratory collapse. The animal was then allowed to recover. The experiment was carried out on four animals and a typical tracing is shown in figure 1.

*Electrocardiographic studies (dog and monkey).* Two monkeys and two dogs under various planes of surgical anesthesia were cardioscoped. No significant abnormalities were observed. After surgical anesthesia of 20 minutes' duration, permanent tracings of the E. C. G. were made on each animal under surgical anesthesia with ethyl vinyl ether. A typical tracing, Lead II, is shown in figure 2 of the monkey before and under surgical anesthesia.

*Effect on the perfused heart (frog).* Ethyl vinyl ether was dissolved in Howell-Ringer's solution and perfused through the frog's heart *in situ*. Solutions containing 25 mg. per cent depressed the rate and amplitude of beat of the heart.

Solutions containing 50 mg. per cent produced cardiac stoppage. The cardiac toxicity is comparable to that of divinyl oxide but more toxic than ethyl ether. A typical tracing from one of three animals is shown in figure 3.

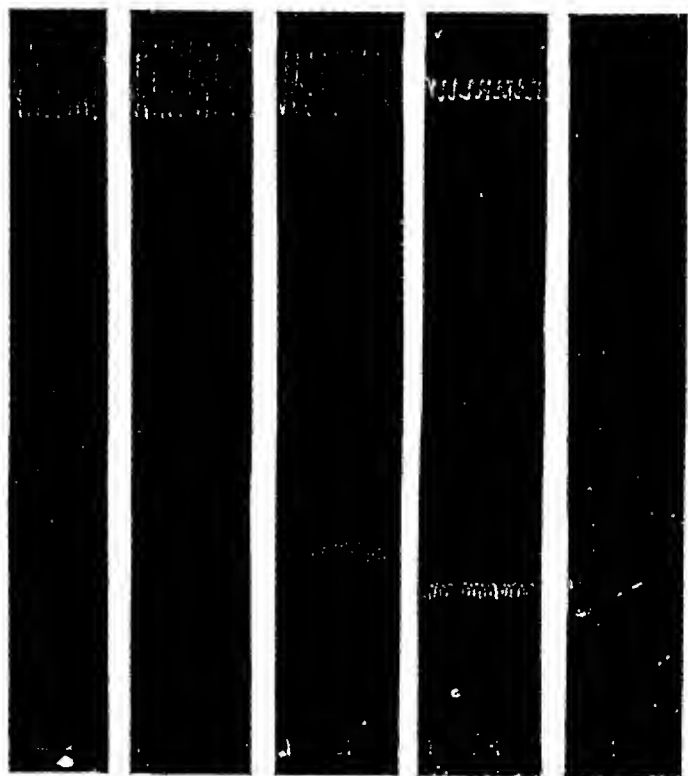


FIG. 1. BLOOD PRESSURE OF DOG UNDER ETHYL VINYL ETHER ANESTHESIA. THE UPPER TRACING IS RESPIRATION. TIME MARKINGS ARE 5 SECONDS

1. Ethyl ether anesthesia.
2. Ethyl vinyl ether anesthesia early stage.
3. Ethyl vinyl ether anesthesia after 30 minutes (deep surgical).
4. Threatened respiratory arrest under ethyl vinyl ether anesthesia.
5. Five minutes after removal of anesthetic cone.

*Liver function tests (monkey and dog).* Two dogs and two monkeys were subjected to the bromsulfalein liver function test as set forth in our studies with cyprethylene ether (6). The dye excretion period was 30 minutes. Twenty-four hours after 60-minute anesthesia with ethyl vinyl ether, the dye excreted was not significantly different from the preanesthetic rate.

The dogs used in the histologic studies of the liver were subjected to a serum amylase determination prior to anesthesia. Before the animals were sacrificed

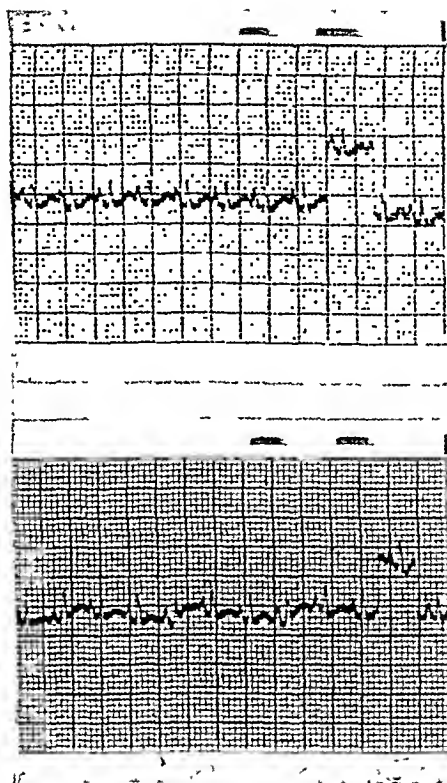


FIG. 2. ELECTROCARDIOGRAMS NORMAL AND UNDER ETHYL VINYL ETHER ANESTHESIA (SURGICAL LEVEL LEAD II)



FIG. 3. EFFECT OF ETHYL VINYL ETHER ON THE FROG'S HEART

1. 25 mg per cent ethyl vinyl ether.
2. Howell-Ringer's solution.

for liver studies the serum amylase was again determined. The serum amylase values remained within normal limits.<sup>1</sup>

<sup>1</sup>We are indebted to Dr Marie A. Andersch, Chief Chemist of the University Hospital, for the amylase determinations in these experiments. Andersch, M. A., J. Biol. Chem., 166, 705, 1946.

*Blood chemistry studies (monkey and dog).* Two dogs and two monkeys were anesthetized with ethyl vinyl ether for 60 minutes. Prior to anesthesia and 24 hours later, blood samples were drawn for analysis. The carbon dioxide combining capacity and blood urea concentration were not significantly altered by this anesthetic experiment in either species.

*Delayed anesthetic deaths (rats).* Twenty adult rats were anesthetized with ethyl vinyl ether to the surgical level and maintained in this state for 30 minutes. Four animals were sacrificed at the end of 2 weeks; no significant findings were observed in the liver or kidneys. At the end of 3 weeks one of the animals had died. The others appeared to be in a healthy condition.

*Histologic studies of viscera (rat, dog and monkey).* Four of the rats used in the delayed anesthetic death studies were sacrificed and their liver and kidneys were found to be free of significant changes. Three dogs were anesthetized for 60 minutes each on 3 alternate days. On the fifth day after the first anesthesia, liver biopsies were performed. There were no significant histological changes observed.

Two *Macacus rhesus* monkeys were subjected to the procedure of repeated anesthetics as applied to the dog. The findings were similar.

*Electroencephalographic studies (dog).* One animal was anesthetized surgically with ethyl ether and electroencephalograms were made from various brain areas. The animal was allowed to awaken and the procedure was repeated using ethyl vinyl ether as the anesthetic. No significant difference could be observed between the electroencephalograms of this animal under the two different anesthetic agents.

*Clotting time and hemolysis (monkey and dog).* The clotting time of blood was determined in two normal monkeys by the capillary tube method. The clotting time was approximately 1 minute. In each animal under anesthesia with ethyl vinyl ether the clotting time of blood increased 10 to 15 per cent.

Volumes of 10 cc. of ethyl vinyl ether in varying concentrations in normal salt solution to which was added 0.1 cc. defibrinated dog's blood were maintained at 25°C. Ten milligrams per cent, 25 mg. per cent and 50 mg. per cent solutions produced no hemolysis over a 24-hour period of observation. During this period a saturated solution of ethyl vinyl ether in normal salt solution produced hemolysis and methemoglobin.

*Precanesthetic medication (dog and monkey).* In monkeys and dogs, inducing ethyl vinyl ether anesthesia with nitrous oxide or cyclopropane-oxygen mixtures was uneventful. Precanesthetic medication with pentobarbital sodium or morphine-atropine was found to be compatible with ethyl vinyl ether anesthesia. Eight experiments were conducted on two monkeys and four dogs.

*Physical properties. Solubility in water.* A 5 cc. volume of ethyl vinyl ether was agitated vigorously with 100 cc. of water for 30 minutes at 23°C. in a "Cassia Flask." The liquids were allowed to separate for 12 hours and the volume of supernatant ether measured. In four experiments the solubility was found to be 0.8 cc.  $\pm$  0.05 per 100 cc. of water.

*Oil/water coefficient.* The oil/water coefficient was calculated from the data of Carr et al. (7) on the relationship between water insolubility and oil/water coefficient. The value for ethyl vinyl ether is  $45 \pm 5$ .

*Inflammability range.* Ethyl vinyl ether is isomeric with cyprome ether (5) and will therefore have the same inflammability range. This is approximately 2.5 per cent for air or oxygen (8).

*Vapor pressure.* The vapor pressure of ethyl vinyl ether determined at 22°C. in a nitrometer is 485 mm.; that of ethyl ether at the same temperature is 471 mm. (9).

#### SUMMARY AND CONCLUSIONS

1. Ethyl vinyl ether, the hybrid molecule between ethyl ether and ethylene, is a volatile liquid exhibiting anesthetic properties when administered by inhalation to various species of animals.

2. The potency of ethyl vinyl ether is approximately twice that of ethyl ether. It is not as potent as divinyl oxide, but requires 1.7 cc./Kg. to produce respiratory arrest in the dog, whereas this value for divinyl oxide is 0.8 cc./Kg. The comparative study of the potency of these three ethers confirms the pharmacologic dictum that potency increases with the degree of unsaturation.

3. In the dog, ethyl vinyl ether anesthesia produces no functional liver damage as shown by the bromsulfalein and serum amylase tests. In these experiments in the rat, dog and monkey anesthetics with ethyl vinyl ether produced no histopathological changes in the liver and kidneys.

4. Neither the monkey's nor the dog's heart showed any significant electrocardiographic changes under anesthesia with ethyl vinyl ether.

5. The blood pressure of the dog remains essentially unchanged under anesthesia with ethyl vinyl ether.

6. This mixed ether compares very favorably with diethyl ether and divinyl ether as an inhalation anesthetic in several species of animals. This first approximation of the anesthetic properties of ethyl vinyl ether, in our opinion, warrants its careful and judicious trial in man by skilled anesthesiologists.

*Addendum.* These experiments having been completed, we deemed that the properties of ethyl vinyl ether warranted its trial in man as an anesthetic. On Tuesday, March 4, 1947, at 10:45 A.M., one of us (J. C. K., Jr.) administered ethyl vinyl ether to an anesthetist, Constance Black, by the open drop method. The induction period was about 60 seconds. Light anesthesia was continued for about 6 minutes. The recovery was rapid and uneventful. The blood pressure and pulse were not significantly altered. The subject stated that the vapors did not irritate the upper respiratory tract.

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# EFFECT OF METRAZOL ON THE ELECTRICAL EXCITABILITY OF THE RESPIRATORY CENTER WHEN DEPRESSED BY PHENOBARBITAL<sup>1</sup>

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There are many reports in the literature on the action of Metrazol as a respiratory stimulant. These reports are quite at variance with one another, some investigators finding positive, and others, negative results. Maloney and Tatum (1) and Veal and Hamilton (2) found that Metrazol, among other drugs, was of no value in counteracting depression due to barbiturates. Maloney (3) working with rats and rabbits narcotized with phenobarbital found that under certain conditions Metrazol, caffeine and coramine may accentuate the depressant action of the barbiturate. Mousel and Essex (4) concluded that Metrazol in lightly anesthetized animals (pentothal) produces some degree of respiratory stimulation; almost no respiratory stimulating effects are seen on animals deeply anesthetized with sodium pentothal and sodium amytal, and that in the more deeply anesthetized animals an increased depression is produced. Barker and Levine (5) found that in cats under light ether anesthesia, Metrazol had no beneficial effect on the cardiorespiratory mechanism, either in normal animals or in those depressed by quinidine, hemorrhage and acid intoxication. Jackson (6), however, reported that while Metrazol failed to produce respiratory stimulation in dogs when deeply anesthetized with ether, when administered to dogs under deep anesthesia produced by evipal, sodium pentothal and sodium amytal, it produces marked specific stimulation of the respiratory center. Jackson used much larger doses of Metrazol than did other investigators, up to 87 mgm. per kgm. With this dosage he observed no convulsions in the dogs anesthetized with barbiturates. Schmidt, Hildebrandt and Krehl (7) reported stimulation of respiration when this has been depressed by morphine.

Recently interest has again been shown in the use of Metrazol in the treatment of acute barbiturate overdosage, as in intravenous pentothal-sodium anesthesia, and as an analeptic to shorten the postoperative period of unconsciousness. Richards, and Piekrell, have reported several cases in which Metrazol was used intravenously when respiration had ceased during pentothal anesthesia (8).

The present study was undertaken for the purpose of determining the action of Metrazol on the respiratory center during deep barbiturate anesthesia, making use of a new criterion for determining the state of activity of the respiratory center, namely, observation of responses to electrical stimulation, as well as rate and tidal air. This technique was used by Wells et al. (9) in a study on picrotoxin in 1944.

<sup>1</sup>Metrazol supplied through the courtesy of Bilhuber-Knoll Corp., Orange, New Jersey.

**METHODS AND RESULTS.** 1. *Intact cats.* Healthy adult cats weighing 2 to 2.5 kgm. were anesthetized with sodium phenobarbital 150 mgm. per kgm., administered intravenously in 10% solution, and a tracheotomy was made. The inspiratory center (10) was then localized by means of a unipolar electrode oriented in the Horsley-Clarke stereotaxic apparatus. The indifferent electrode was inserted in the rectum. A Goodwin thyatron type stimulator discharging on a frequency of 1000 per second, a falling phase of 6 sigma, delivered the stimuli. The least voltage required to produce and maintain an inspiratory cramp (threshold stimulus) varied between 0.2 volts to 0.7 volts in the different animals. The inspiratory response produced by the threshold stimulus was in every case much less than that produced by stimuli of higher voltage. The tracheotomy tube was then connected to a closed circuit containing oxygen, which was replenished at a constant rate. The circuit included a soda-lime tube for the removal of carbon-dioxide and a small Krogh-type spirometer of 400 cc. capacity, graduated in 5 cc. units, and equipped with an ink writing point for making tracings of the respiratory movements on a continuously moving kymograph.

As controls, eight cats were prepared in the above manner, and a period of normal breathing was recorded. During this time the threshold stimulus was determined. A second, or "depressant" dose of sodium phenobarbital was then given intravenously. This dose varied from 65 to 135 mgm. per kgm. Response to electrical stimulation of the center, and readings of tidal air and rate were taken at intervals, until the cat died, or until it was killed after not less than one hour and twenty minutes following the depressant dose of sodium phenobarbital.

Figure 1 reproduces a typical kymogram and graph illustrating the results obtained in this control group of cats. These animals showed no appreciable spontaneous recovery of respiratory activity during the period observed.

An experimental series of eight cats, similarly prepared, was treated with varying doses of Metrazol during the depression caused by the "depressant" dose of sodium phenobarbital. This dose of phenobarbital was 90 mgm. per kgm. in five cases, 80, 135 and 160 mgm. per kgm. in each of the other cases. The dosage of Metrazol varied from 50 to 300 mgm. per kgm., administered intravenously in 10% solution.

The administration of Metrazol intravenously in large doses causes a prompt onset of increased activity and excitability of the depressed inspiratory center. This occurs within one minute after giving the Metrazol. This is clearly illustrated in figure 2, which also shows that this response is fairly persistent.

One cat was prepared in like manner, but this time with the electrode placed in the expiratory portion of the respiratory center (10). A voltage of 0.9 volts was found to produce forced expiratory movements, which were held for the duration of the stimulus (eight seconds). After a few normal readings, a depressant dose of sodium phenobarbital was administered (90 mgm. per kgm.). When an adequate depression of the expiratory center was attained, as indicated by decreased rate and electrical excitability, Metrazol in a dose of 200 mgm. per kgm. was given.

The results obtained showed complete and sustained recovery of the response to electrical stimulation. There was temporary recovery of rate, lasting only six minutes. The tidal air, however, shows a sustained recovery to more than the original figure.

A short (0.5 to 1 minute) period of apnea was observed after four of twelve

doses of Metrazol given to the intact animals. No convulsions were seen in any of this series.

To study the possibility that blood pressure changes might be responsible for the respiratory responses observed, two intact cats were prepared as already described. A femoral artery was cannulated in order to record blood pressure changes by means of a mercury manometer. Fastusol pink, 100 mgm. per kgm.

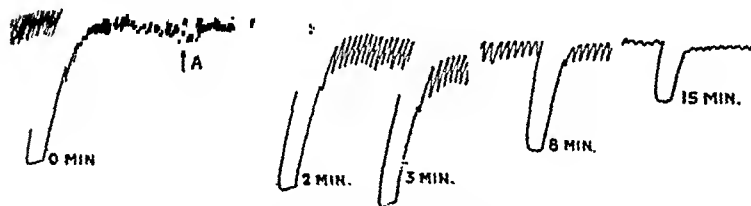
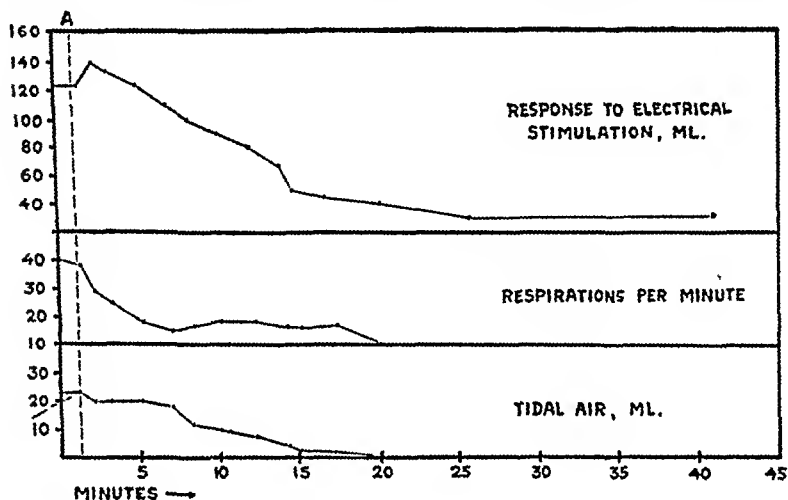


FIG. 1. INTACT CONTROL. ANESTHESIA—SODIUM PHENOBARBITAL 150 MGm. PER KGm. I.V. STIMULUS CONSTANT AT 0.7 V. THROUGHOUT

At A, a depressant dose of phenobarbital (78 mgm. per Kg.) was administered intravenously. The subsequent changes in respiration are illustrated by the graphs and the related segments of kymographic record. The deep inspiration shown by the long downstroke of the kymogram.

was used as an anticoagulant. Each cat received depressant doses of phenobarbital, 60 mgm. per kgm., and doses of Metrazol of 200 mgm. per kgm. These cats showed typical respiratory changes, with fall in blood pressure accompanying both the injection of phenobarbital and Metrazol. The apnea, observed after one of these injections, was concurrent with the lowest level of blood pressure obtained. Recovery of blood pressure and of respiratory movements occurred synchronously. Phenobarbital produced a profound fall in blood pressure, and

apnea. Metrazol was then given, following which there was recovery of both respiratory activity and blood pressure. A second dose of Metrazol at this time produced a temporary fall of blood pressure, accompanied by a short period of apnea. A second dose of phenobarbital produced another fall of blood pressure and respiratory depression, and at this time 14 units of Pitressin was administered. This produced a rise of blood pressure to its normal value, but the respiratory depression continued unabated, and even increased. The Pitressin effect soon passed, and the inspiratory center was stimulated, producing a response much greater than the tidal air at the time, which was only about 5 cc.

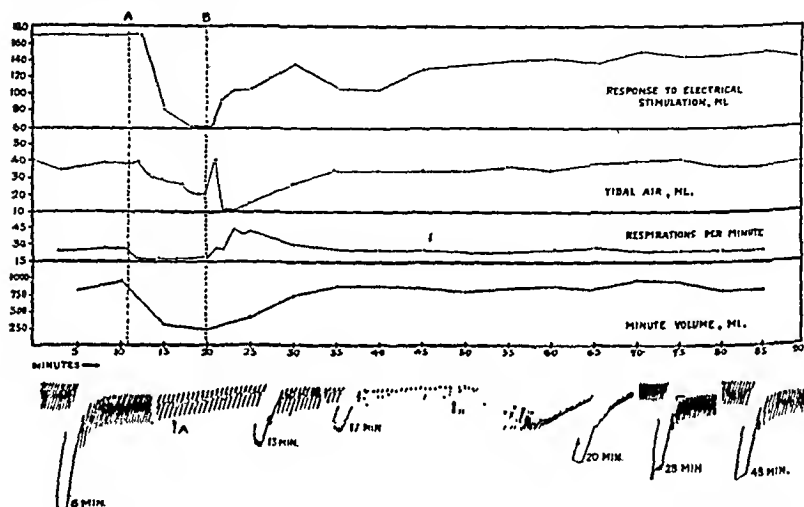


FIG. 2. INTACT CAT. ANESTHESIA—SODIUM PHENOBARBITAL 150 MG. PER KG. I.V. STIMULATING ELECTRODE IN INSPIRATORY CENTER. STIMULUS CONSTANT AT 0.6 V. THROUGHOUT

At A, 90 mgm. per Kg. of sodium phenobarbital were administered I.V. The resulting depression of respiratory activity is indicated in the graphs. At B, 200 mgm. per Kg. of Metrazol were given I.V. The improvement in the respiratory condition of the animal is evident. The segments of kymograph record below show response typical of those seen throughout the experiment.

This inspiratory movement was then promptly followed by a rise in blood pressure to a normal value, later falling again. A second stimulation produced a similar effect.

2. *Decerebrate cats.* It was now desired to test the effect of Metrazol on the respiratory center under various other conditions: (1) in the absence of any depressant agent; (2) in the absence of any Metrazol effect on higher centers; (3) in the presence of a depressant agent but with the respiratory center isolated from all higher centers.

For this purpose decerebrate cats were prepared by the method of Davis and Pollock (11). Both common and external carotid arteries were ligated, as was also the cord-like structure representing the internal carotids. The basilar

artery was then exposed by an oral approach and was likewise ligated. The animals were under light ether anesthesia during the surgical procedure. This method produces decerebration in the midpons, at the level of the point of exit of the 5th cranial nerve, and with a minimum of trauma to the pontine and medullary structures. The cats were then prepared for recording respiratory activity as in the intact animals.

As controls for the decerebrate series, two decerebrate cats received single doses of phenobarbital, 50 and 75 mgm. per kgm., and were observed for 60 to 90 minutes following the injection.

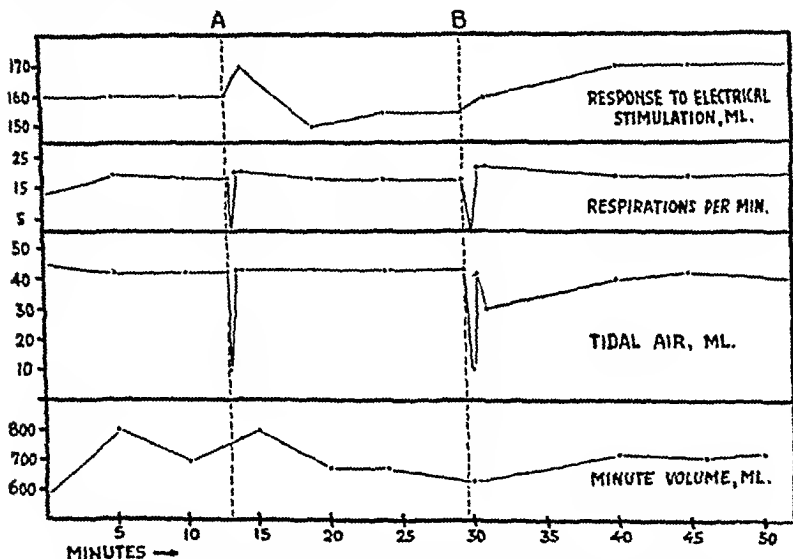


FIG. 3. DECEREBRATE CAT. NO PHENOBARBITAL. STIMULATING ELECTRODE IN INSPIRATORY CENTER. STIMULUS CONSTANT AT 0.3 V. THROUGHOUT

AT A, 50 mgm. per Kgm. and at B, 100 mgm. per Kgm. of Metrazol were given intravenously. No sustained change in respiratory activity was noted.

The results in these cats are comparable to those in the intact control series. A profound and prolonged depression of all respiratory activity was produced by the phenobarbital.

Five cats, after being decerebrated and a period of normal respiration recorded, were treated with Metrazol in dose varying between 25 and 267 mgm. per kgm. Reaction to electrical stimulation was then determined at approximately five minute intervals thereafter. Most of these cats received two injections of Metrazol.

Figure 3 is illustrative of the results obtained with this undepressed, decerebrate series. It is evident that there is no sustained, marked change in the minute volume, rate, tidal air or electrical excitability of the respiratory center. A period of apnea as already described was observed eight times out of ten doses

of Metrazol. This apnea was inspiratory once, in mid-position of the thorax three times, and expiratory four times. Slight or moderate convulsions occurred after only three of the doses of Metrazol.

Seven decerebrate cats received sodium phenobarbital in doses ranging from 50 to 125 mgm. per kgm., intravenously. Metrazol, 50 to 200 mgm. per kgm., was then administered after respiratory depression was quite apparent. Readings of the respiratory activity were taken as in previous examples.

The results obtained in one of this depressed, decerebrate series are illustrated in figure 4. These findings were typical of this series. Recovery of respiratory center activity, as measured by rate, tidal air and electrical excitability, is considerable. Of thirteen doses of Metrazol given, varying from 50 to 200 mgm.

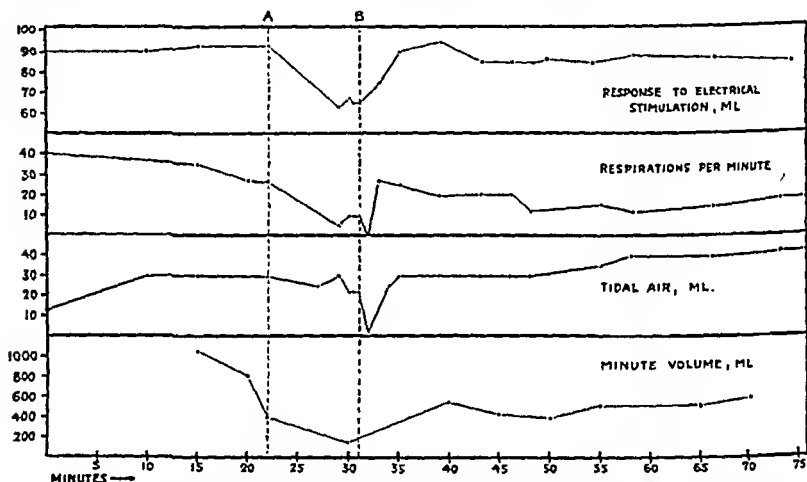


FIG. 4. DECEREBRATE CAT. STIMULATING ELECTRODE IN INSPIRATORY CENTER. STIMULUS CONSTANT AT 0.3 V. THROUGHOUT

At A, cat received 90 mgm. per Kgm. Sodium Phenobarbital intravenously. The respiratory depression is evident. At B, Metrazol 100 mgm. per Kgm. given intravenously produced typical recovery of respiratory rate, tidal air and response to electrical stimulation.

per kgm., six were followed immediately by apnea as previously described, before any stimulation was seen, and seven showed immediate respiratory stimulation. There were no convulsions in any of this group.

The inspiratory center alone was tested in all the decerebrate series.

DISCUSSION. The results of these experiments indicate that Metrazol, administered in large doses to cats depressed by phenobarbital, produces a considerable recovery of respiratory activity, both in the inspiratory and expiratory portions of the respiratory center. It is to be noted that those investigators who reported no stimulation of respiration by Metrazol were using much smaller doses, 5 to 20 mgm. per kgm. It was this observation that prompted the use of larger doses. Larger doses in dogs had been reported by Jackson to produce stimulation of respiration. The use of large doses we believe is justified by the fact that the dosage of the barbiturates is also greatly in excess of that usually

administered. Despite the large amount of Metrazol given, convulsions were not seen in any cat that had received sodium phenobarbital, and in only three of the undepressed decerebrate cats. These findings confirm the report by Jackson (6) that Metrazol produced respiratory stimulation, but no convulsions, in dogs deeply anesthetized with various barbiturates. Jackson used doses up to 87 mgm. per kgm. in these dogs, and of more than 100 mgm. per kgm. in dogs anesthetized with avertin.

As already mentioned, the decerebrate series were run for the purpose of studying the effect of Metrazol in the absence of any depressant drug, and in animals free of any Metrazol effect on the higher centers.

Pitts, in rebreathing experiments on cats obtained evidence that in a decerebrate preparation the mechanism for increasing the depth and rate of respiration are intact, and direct chemical stimulation of the respiratory center is not interfered with (12). Thus, in the decerebrate series all higher centers whose connection with the pons and medulla could possibly affect the responses of the respiratory mechanism have been removed, while this mechanism itself is left intact. In addition to removing influences from higher nervous centers, the technique of decerebration used also eliminates all possible carotid sinus and carotid body influence on the respiratory center.

The results with undepressed, decerebrate cats treated with Metrazol are quite different from those obtained in intact cats, whose respiration had been depressed by phenobarbital. The latter show a remarkable increase of respiratory activity over the depressed level. The effect on respiration in decerebrate cats is inconstant—stimulation of short duration in some instances; in others an actual depression of activity is produced. A significant, prolonged stimulation of respiratory activity cannot be said to occur.

A picture very different from the above is seen in the case of decerebrate cats that have received a dose of phenobarbital sufficient to depress the respiratory center. The increase in activity in some instances is several times greater than the activity and excitability of the center at the time the Metrazol was given. Comparing figures 2 and 4, the similarity between the two depressed series is easily seen. The findings in the depressed series, both intact and decerebrate, are in agreement with those of Jackson in dogs under deep barbiturate anesthesia. Our findings in undepressed, decerebrate cats resemble Jackson's results in dogs under ether anesthesia, which showed convulsions but no respiratory stimulation. Similar results were obtained by Barker and Levine in cats under light ether anesthesia—convulsions, but no respiratory stimulation. Schmidt and Hildebrandt (7) believed the stimulation of respiration depressed by morphine was due to strong cortical action of the Metrazol. This, of course, is ruled out in the decerebrate preparations. It therefore seems likely that the greater part of the stimulatory action of Metrazol on the respiration depends on the presence of an actual depression, particularly that produced by barbituric acid derivatives.

Barker and Levine in 1928 (3) and Hildebrandt (13) in 1936, comment on the short period of apnea following large doses of Metrazol. In our experiments it was observed that this period of apnea occurred in 80% of cases in the undepressed decerebrate series, in 46.1% of the depressed decerebrate series.



Convulsions occurred only in the undepressed decerebrate series, in three of five animals, and no increase in the normal decerebrate rigidity was noted after the Metrazol injections in any of the other decerebrate cases. In the series of intact cats, only 30% of the doses administered were followed by periods of apnea, and none by convulsions. A rough parallelism is thus seen between the occurrence of apnea and convulsions in these animals, and the absence of phenobarbital. It is therefore believed that the apnea is due to an initial toxic action of the Metrazol, possibly an overstimulation of both the inspiratory and expiratory portions of the respiratory center, with the production of a cramp, this action being modified or prevented in the presence of the phenobarbital depression. Recording of action-potential changes in phrenic and intercostal nerve preparations will serve to help clarify this point.

A marked rise in blood pressure, of short duration, might be also the cause of the period of apnea. Such a rise was described by Hildebrandt, and by Gavidia (14) in curarized dogs. Barker and Levine, however, reported a fall in blood pressure following administration of Metrazol in intact ether-anesthetized cats. In the two intact phenobarbitalized cats on which blood pressure tracings were made, a marked fall in blood pressure was observed following the administration of Metrazol. The apnea was concurrent with the lowest level of the blood pressure. The pressure rose to normal within seven minutes.

As the increased respiratory action seen when Metrazol was administered to the cats depressed by phenobarbital was accompanied by recovery of blood pressure, the question therefore arose: was the respiratory stimulation seen dependent on, or due to, a recovery of blood pressure? Rise in blood pressure alone could conceivably, by improving the blood supply to the medulla, thereby improve respiratory function. That this was not the case is shown by the cat receiving Pitressin when the respiratory activity and blood pressure had been depressed by phenobarbital. Although recovery of blood pressure to a normal value was prompt following the Pitressin, there was no concomitant increase in respiratory activity. The blood pressure, which soon fell again to the depressed level, rose immediately, almost precipitously, to the normal when the respiratory center was stimulated. This stimulation produced an inspiratory movement of many times the volume of the tidal air, which was quite depressed. Following this stimulation the tidal air was improved for two minutes, during which time the blood pressure remained at a fair level. As soon as the tidal air decreased the blood pressure dropped rapidly. A second electrical stimulation produced another good inspiratory movement but no improvement of tidal air. The blood pressure again rose to a nearly normal level following this inspiratory movement. It is thus clear that at least in this case the recovery from a low blood pressure due to phenobarbital is dependent on adequate ventilation of the lungs rather than vice versa.

#### SUMMARY AND CONCLUSIONS

Intact cats, anesthetized with sodium phenobarbital, and decerebrate cats, received large intravenous doses of Metrazol after the respiratory activity had

been depressed with phenobarbital. These cats showed considerable recovery of respiratory activity.

Decerebrate cats, with normal respiratory center, showed no consistent stimulation of respiratory activity following Metrazol administration.

It is believed that a major portion of the stimulation observed in the two depressed series is due to a pharmacological antagonism to the barbiturate.

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## THE TRACHEAL CHAIN

### I. A PREPARATION FOR THE STUDY OF ANTISPASMODICS WITH PARTICULAR REFERENCE TO BRONCHODILATOR DRUGS

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The purpose of this communication is to present a method for the study of the antispasmodic action of drugs on the tracheal musculature. The method is based upon the finding that the excised trachea of the guinea pig will respond to many drugs with the characteristic actions for which the drugs are well known, and that with proper magnification, the response can be recorded and measured for comparative purposes. Although the method is suitable for the study of antispasmodic drugs in general, emphasis is given to its use in the testing of bronchodilators, because of the close anatomical and physiological association which exists between tracheal and bronchial musculature.

Trendelenburg (1) studying the action of drugs on the bronchi, observed the behavior of strips of bronchial muscle dissected from the ox. Macht and Giu-Ching Ting (2) used excised strips of surviving muscle from the bronchi of pigs. These workers used muscle obtained from large animals because the actual mechanical changes in the muscle are quite minute. In our search for a simple screening test for bronchodilator action, it was found that a small laboratory animal as the guinea pig could be satisfactorily used by sectioning the primary bronchi of four or five animals into circular rings about 1 mm. wide and connecting the rings in chain-fashion with loops of silk thread. A chain of 10 or 12 rings produced such an additive effect that the constriction or dilatation produced by various drugs could be clearly demonstrated. However, in view of the fact that the bronchi of the guinea pig are very short and totally embedded in lung tissue, thus requiring elaborate dissection and the use of several animals to obtain a single preparation, it was decided to study the behavior of a chain of rings from the much longer and more easily dissected trachea. As was to be expected on the basis of the physiological anatomy which is common to both tracheal and bronchial musculature, a chain of 12 rings obtained from the entire trachea of one guinea pig was found to react to spasmogenic and antispasmodic drugs in a manner quite similar to that of a chain of bronchial rings obtained from several guinea pigs.

*The Tracheal Chain Preparation.* An adult guinea pig is killed by a blow on the head. The trachea is removed and sectioned with a pair of scissors into 12 rings of approximately the same width. The rings are kept moist with Ringer's solution while they are connected in series by means of short loops of silk thread.

*Experimental.* The chain is mounted in a bath of Van Dyke-Hastings' solution<sup>1</sup> (3)

<sup>1</sup>The Van Dyke-Hastings' solution was modified by the addition of dextrose to make 0.05 per cent, and before being used it was saturated with the CO<sub>2</sub>-O<sub>2</sub> gas mixture under a layer of heavy liquid petrolatum.

maintained at 37.5°C and aerated by bubbling a mixture of 5% CO<sub>2</sub> and 95% O<sub>2</sub> through a side tube similar to that described by Harne (4). Washing is conducted by flooding upward from the bottom to avoid exposure to the air. Although ink-writing devices may be used, the studies described in this paper were made with a light, sensitive, 13-inch muscle lever yielding twelve-fold magnification. A short piece of aluminum wire coiled loosely about the end of the lever formed an almost frictionless writing point when used with lightly smoked paper. The kymograph was set to move at the rate of 0.1 cm. per minute.

The only tension required for the preparation is that needed to keep the chain vertical because the muscle fibers which stretch across the ends of the C-shaped ring of cartilage apparently are normally under a state of tension, for if they are sectioned, the ring springs open. It was found that satisfactory tension could be obtained as follows: The lever is first balanced by hanging small weights on the short arm. Then sufficient of these weights are removed to equal the weight of the chain. Finally the preparation is connected. For example, if the chain weighs 350 mgm., weights totaling 350 mgm. are removed. Under these conditions, the only tension used is that caused by the weight loss of the chain through the buoyancy of the bathing solution.

In studying the antispasmodic action of a drug, we observed first its effect on the normal or untreated trachea (see figure 1). Then its spasmolytic action was determined by its efficacy in relieving the spasms induced respectively by histamine phosphate, acetylcholine bromide and barium chloride (see figure 2).

**RESULTS.** In table 1 are summarized the results obtained on 72 guinea pig tracheas. The drug dilutions listed represent the dilutions of the antispasmodics which in typical experiments were found to produce a definite practical effect as defined in the legend of the table.

*A Comparison of Bronchodilator and Spasmolytic Drugs on the Untreated Trachea.* The three well-known bronchodilators, epinephrine, aminophylline and papaverine dilated the untreated trachea. In sharp contrast, the spasmolytics, atropine, Novatropine, Syntropan and Trasentin and the antihistamine drug, Benadryl, produced no relaxation (see figure 1). In fact, Benadryl in large doses caused contraction. Figure 1 also shows that epinephrine caused prompt relaxation but that after washing, its action was brief as shown by the rapid return to the original level. Aminophylline was much less potent from a dosage standpoint, as might be expected from clinical experience, but it was nevertheless promptly effective. Papaverine acted the most slowly, but its effect was quite prolonged, even after washing. It was found that graded responses could be obtained with graded doses of these three bronchodilator drugs. Work is in progress on the development of assays for bronchodilator drugs.

*The Antagonism of the Bronchodilators toward Spasmogenic Drugs.* All three bronchodilators counteracted the spasmogenic drugs, histamine, acetylcholine and barium chloride, probably by virtue of their ability to relax tracheal muscle rather than by any specific drug antagonism. That is, the effect obtained may be considered the resultant of two drugs acting independently but in opposite directions. As figure 2 shows, epinephrine was rapid but evanescent in action, even without washing. Aminophylline was also rapid in onset, but its effects were more prolonged and papaverine was both slow in onset and of long duration. Table 1 shows the very wide difference in the potencies of these three bronchodilators.

*Atropine.* This drug produced no visible effect of its own on the tracheal muscle. In sufficiently high concentrations (1:50,000), it inhibited the action

TABLE 1

Maximal dilutions of the antispasmodics that under the conditions described in this paper produced (I), a dilatation of the normal or untreated trachea sufficient to cause approximately a 1 cm. fall in the tracing, and (II), a definite relief (75 to 100 per cent) of the constrictions induced by histamine, acetylcholine and barium chloride

ANTISPASMODIC	I	II		
	Normal Muscle	Histamine Phosphate 1:500,000	Acetylcholine Bromide 1:1,000,000	Barium Chloride 1:5000
Epinephrine	1:100,000,000	1:20,000,000	1:20,000,000	1:80,000,000
Aminophylline	1:200,000	1:25,000	1:5000	1:20,000
Papaverine	1:2,000,000	1:500,000	1:400,000	1:500,000
Atropine Sulfate	None	1:50,000	1:50,000,000	None
Novatropine	None	None	1:20,000,000	None
Syntropan	None	None	1:200,000	None
Trasentin	None	None	1:125,000	None
Benadryl	None	1:15,000,000	1:250,000	None

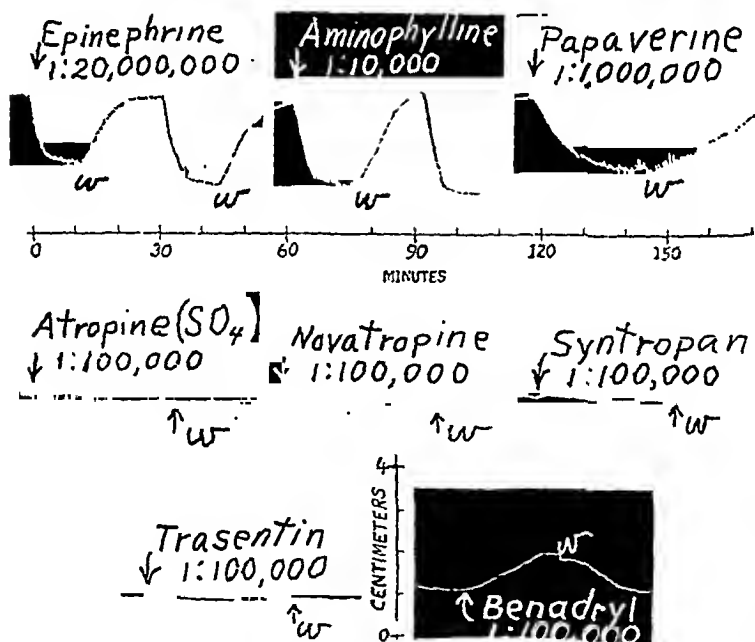


FIG. 1. THE EFFECT OF THE INDICATED CONCENTRATIONS OF THE ANTISPASMODICS ON THE NORMAL OR UNTREATED TRACHEAL CHAIN. (W) INDICATES WASHING

of histamine but it was found to be a thousand times more potent in counter-acting acetylcholine (see table 1). Atropine failed to alleviate the spasms of

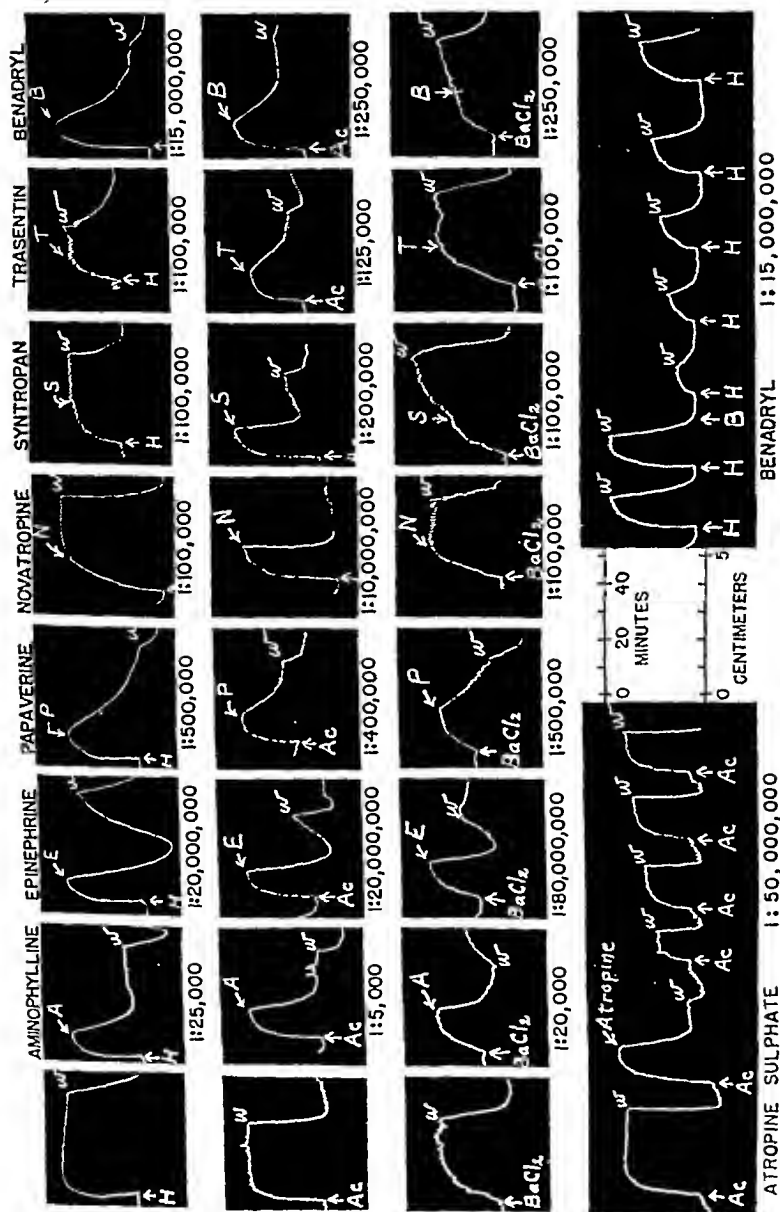


FIG. 2. THE EFFECT OF THE INDICATED CONCENTRATIONS OF THE VARIOUS ANTISPASMODICS ON THE SPASMS INDUCED BY (H) HISTAMINE PHOSPHATE 1:500,000, (Ac) ACETYLCHOLINE BROMIDE 1:1,000,000, AND BARIUM CHLORIDE 1:5000 ON THE TRACHEAL CHAIN. (W) INDICATES WASHING

barium chloride. Figure 2 illustrates not only the ability of atropine to relieve the constriction of acetylcholine but also its residual inhibiting action even after several washings.

*Novatropine.* Novatropine resembled atropine in its failure to relax the untreated muscle and its inability to counteract barium chloride spasms. It differed from atropine in these experiments in failing to relax histamine-constricted muscle (see figure 2). This drug was found to be less active than atropine in antagonizing acetylcholine contraction and its residual inhibiting action after washing was of much shorter duration than that obtained with atropine.

*Syntropan and Trasentin.* These two drugs showed no dilator action on the untreated trachea and as table 1 shows, they resembled Novatropine in all respects except that Novatropine was found to be more potent in antagonizing acetylcholine. The failure of these two drugs to relieve histamine spasm in the guinea pig trachea is in agreement with the finding of Loew and his collaborators (5) that Syntropan and Trasentin are ineffective in relieving histamine-induced bronchoconstriction in guinea pigs (see figure 2).

*Benadryl.* Benadryl caused no dilatation of the untreated trachea but was found to possess powerful anti-histamine action. Its marked preferential action against histamine resembles that of atropine against acetylcholine (see table 1). This drug was also found to have definite atropine-like action against acetylcholine. No relaxation of the barium chloride contraction was obtained with Benadryl, despite the fact that this drug has been found effective in antagonizing barium chloride spasm on isolated guinea pig ileum (6) (see figure 2). In figure 2, lower tier, a technique for studying the more specific types of antagonisms is illustrated. In this case, the antihistamine action of Benadryl is demonstrated by its ability to prevent the constricting action of histamine. Its residual inhibiting action even after several washings is also shown.

**DISCUSSION.** As the various tracings presented in this paper show, the tracheal chain did not exhibit spontaneous contractions. The few irregularities shown seemed to be due to air currents in the room and to vibrations of the laboratory table. Preparations were frequently found to survive 12 hours or more unless the tissue was damaged by the drug being studied. No particular precautions in selecting guinea pigs, with regard to sex or weight, were found to be necessary and almost every pig used furnished a satisfactory preparation.

The long-sustained submaximal contractions of the tracheal chain produced by spasmogenic agents facilitate the study of spasmolytic drugs with respect to both onset and duration of action. Although the tracheal muscle behaves like intestinal muscle in many ways there are also some differences in its behavior to certain spasmolytic drugs. Trasentin and Syntropan have been found to antagonize the spasmogenic action of histamine on the isolated intestine (5) (6) (7) but we have been unable to demonstrate any anti-histamine action on the tracheal muscle with these two drugs. Also, although aminophylline has been reported (5) (8) to be relatively ineffective in counteracting the action of histamine on intestinal smooth muscle, we have found it to be quite active in relaxing the histamine-constricted tracheal musculature. This may be related to the

fact that aminophylline relaxes the untreated trachea while it has been stated to cause transient spasms of the isolated gut (8) (9).

Although this paper is concerned with the action of drugs on the trachea, we have used the term bronchodilator in connection with epinephrine, aminophylline and papaverine because these drugs are so-called in common usage. It is recognized that a given drug may act differently on the bronchioles, the larger bronchi or the trachea (10). The available evidence, however, indicates that the trachea and the larger bronchi react much alike to drugs. Histologically speaking, the trachea and the bronchi possess a common type of cartilage and muscle. Pharmacologically speaking, Florey and Wells (11) reported that both the trachea and the bronchi of the cat react in the same way toward epinephrine, atropine and pilocarpine. Also Isogawa (12) found that histamine caused simultaneous constriction of both the trachea and the bronchi of the guinea pig. The results reported in this paper are in accordance with the clinical behavior of epinephrine and aminophylline, as bronchodilator drugs.

#### SUMMARY

1. A technique is described for recording the constrictions and dilatations of the excised guinea pig trachea when exposed to drugs.

2. The tracheal muscle of the guinea pig has been found a suitable preparation for the study of antispasmodic drugs. The long sustained contractions produced by the spasmogenic agents were found to facilitate the study of both onset of action and duration of the effect of the spasmolytic drug.

3. The guinea pig trachea was found to exhibit good smooth muscle reactions similar to those of the bronchi. Well-known bronchodilators as epinephrine, aminophylline and papaverine were found to dilate the normal or untreated trachea and all three drugs relieved the spasms induced by histamine, acetylcholine and barium chloride.

4. Atropine, Novatropine, Syntropan, Trasentin and Benadryl did not dilate the normal or untreated trachea, failed to relieve barium chloride spasm but were found to counteract acetylcholine action. Of this group of drugs only atropine and Benadryl were effective against histamine, the latter being outstanding in this respect.

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# THE PHARMACOLOGY OF N-ALKYL HOMOLOGUES OF EPINEPHRINE

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Konzett (1a, b) has described the pharmacologic actions of homologues of epinephrine in which the methyl group on the N was replaced by ethyl, propyl, isopropyl, butyl, and isobutyl groups. The results obtained suggest that the N-ethyl homologue most nearly resembles epinephrine in its pharmacologic effect and that with larger N-alkyl groups, epinephrine-like action is greatly modified. In a previous publication, Lands, Rickards, Nash and Hooper (2) have described the results obtained with a similar series but in which the sympathomimetic nucleus was 1-(p-hydroxyphenyl)-2-aminoethanol, and in which the N-alkyl substitutions included secondary and tertiary butyl groups. These two latter derivatives, like the N-isopropyl derivative, were found to be very active vasodepressor agents. More recently we have investigated the pharmacology of the N-sec. butyl homologue of epinephrine and compared it with the corresponding N-isopropyl homologue (ISUPREL).<sup>1</sup> The results obtained are described in this communication.

**EFFECT ON THE HEART AND CIRCULATION.** Cardiac effects were determined on isolated perfused hearts of frogs and of rabbits. In experiments on the frog, the brain and spinal cord were pithed and a cannula inserted into the sinus venosus. Frog Ringer solution was passed through the heart at constant pressure and the excess fluid allowed to drain away from the cut arteries. All drugs were dissolved in frog Ringer solution and injected directly into the perfusion stream near the heart. Five to ten micrograms of Isuprel or the N-sec. butyl (0-4, 1424) derivative increased heart action. These effects were more prolonged than those caused by epinephrine. However, one difference in response should be pointed out. With the injection of ten micrograms or more of epinephrine, the heart was brought to diastolic standstill, whereas, with an equivalent amount of Isuprel or the N-sec. butyl derivative, no such effect was observed.

The isolated rabbit heart was perfused according to the method of Langendorf. As described above, the injections were made directly into the perfusion stream near the heart. Representative results are shown in figure 1. The results obtained indicate that 0.2 to 2.0 micrograms of Isuprel or the N-sec. butyl derivative stimulate the heart, increasing both rate and amplitude.

In a few experiments on anesthetized dogs, myocardiographic recordings of the left ventricle were made according to the method of Cushny. In order to

<sup>1</sup> This compound is being distributed for clinical investigation under the name of Isuprel by Frederick Stearns & Co., Division of Sterling Drug, Inc.

prevent reflex stimulation of the heart resulting from a fall in blood pressure, a small amount of 'Neo-Synephrine' HCl was injected with the depressor drugs; the amount used had little or no effect on the heart beat. Both Isuprel and the

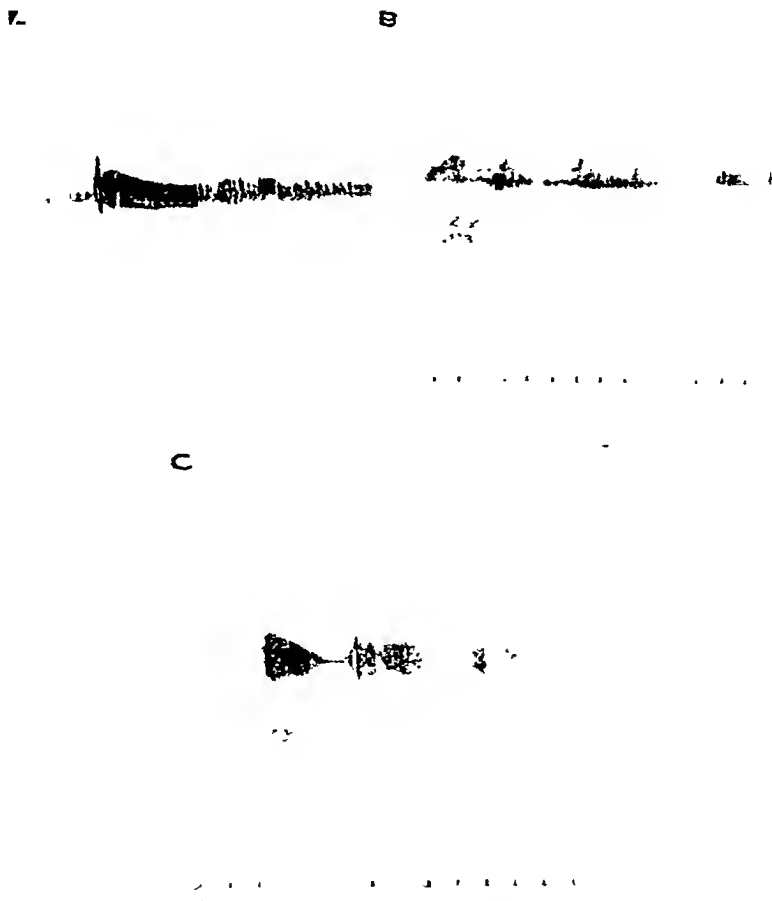


FIG. 1. EFFECT ON THE ISOLATED PERFUSED RABBIT HEART

Drugs introduced directly into the perfusion cannula.

A. Isuprel HCl—0.2 micrograms

B. N-sec. butyl derivative—2.0 micrograms

C. Epinephrine—0.2 micrograms.

Time intervals 5 seconds.

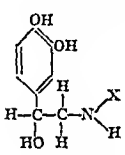
N-sec. butyl derivative caused a prompt increase in both rate and amplitude in the absence of blood pressure changes and these cardiac changes lasted for approximately twenty minutes.

The effect of the two derivatives on carotid blood pressure was determined

in dogs anesthetized with sodium pentobarbital. The above compounds were administered intravenously, intramuscularly, or injected directly into the lumen of the small intestine. Results obtained are shown in table 1. Intravenous injections of Isuprel caused a marked fall in blood pressure with doses of 1-2 micrograms/kgm. (figure 2a). Direct comparison indicates that this fall is of somewhat greater magnitude and duration than the rise in blood pressure obtained with an equal dose of epinephrine. The intravenous administration of the N-sec. butyl derivative indicates that this substance is somewhat less

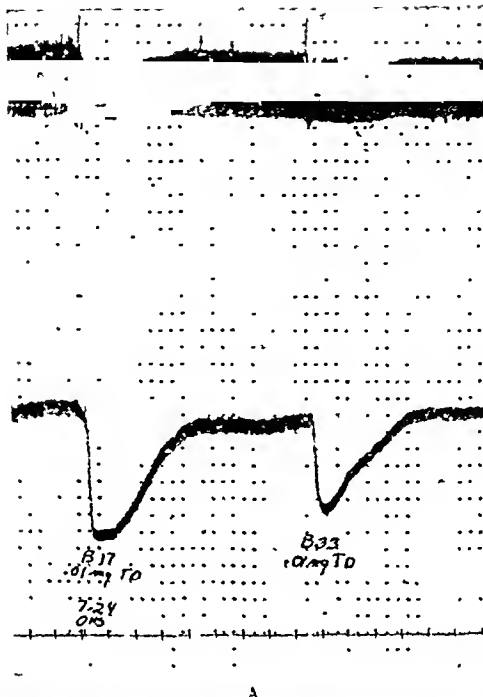
TABLE 1

*Effect on mean carotid blood pressure of the dog. Acute toxicity in mice*

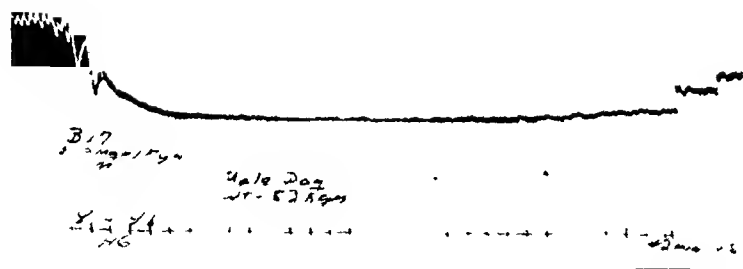
 COMPOUND	X	MODE OF ADMINISTRATION	DOSE PER KGm.	MAXIMUM CHANGE IN BLOOD PRESSURE	DURATION OF SOME CHANGE IN BLOOD PRESSURE	TOXICITY	
						Intraperitoneal in albino mice	
						No. of mice	Results
Isuprel Hydrochloride	isopropyl	intravenous	0.6 microgram	mm. Hg -38	minutes 2-4	203	L.D. 0 420
		intravenous	1.0 microgram	-41	3-12		L.D. 50 450
		intramuscular	0.10 mgm.	-43	prolonged (51- >205)		L.D. 100 >550
		intraintestinal	0.25 mgm.	-44	prolonged (>50)		
0-4, 1424 Acetate	secondary butyl	intravenous	1.0 microgram	-33	2-4	75	L.D. 0 400
		intravenous	2.4 microgram	-37	3-5		L.D. 50 450
		intramuscular	0.10 mgm	-35	prolonged (32- >123)		
		intraintestinal	0.25 mgm.	-35	prolonged (>48)		L.D. 100 >520
Epinephrine H <sub>2</sub> drochloride*						34	L.D. 0 3 L.D. 50 4 L.D. 100 5

\* Prepared as described in the Pharmacopeia of the United States—Twelfth Revision. Dose indicates the amount of 1-epinephrine base

depressor than Isuprel. This difference in effectiveness is still apparent when allowance is made for the difference in molecular weights. Both of the above compounds cause a prolonged fall in blood pressure when administered intramuscularly in doses of 0.1 to 0.2 mgm./kgm. (figure 2b). As indicated above, this is accompanied by a marked increase in heart rate. Similar results were obtained when these substances were injected directly into the small intestine of the anesthetized dog. Absorption from this site appears to be rapid, effects on blood pressure following administration being apparent as quickly as those following intramuscular injection. Approximately two and one-half times as much of the compound must be placed into the intestine to produce equivalent falls in blood pressure.



A



B

FIG 2 EFFECT ON CAROTID BLOOD PRESSURE OF ANESTHETIZED DOGS

A Effect of Isuprel HCl (B 17) and the N sec butyl derivative (B 33) in an intravenous dose of 1.0 microgram/kgm

B. Effect of Isuprel HCl in an intramuscular dose of 0.15 mgm /kgm The kymograph was stopped for 42 and 18 minutes, as indicated, near the end of the record Time intervals in minutes.

Systolic blood pressure was determined in unanesthetized dogs by means of a mercury or aneroid manometer to which was attached a cuff specially designed to fit snugly about the thigh. In most dogs, a strong pulse may be obtained from the anterior tibial artery along the dorsal surface of the foot and, in these, systolic pressure may be determined by palpation. The oral administration of 0.1 to 0.5 mgm./kgm. of Isuprel caused a marked tachycardia which developed rapidly following the administration of the drug. Associated with this increase in heart rate, there was usually a rise in systolic pressure. Although pressure rose, there was evidence of peripheral vasodilatation, inasmuch as unpigmented skin areas became quite pink. As the cardiac effect of the drug diminished, blood pressure rapidly returned to normal and, in most experiments, continued

TABLE 2  
*Effect on systolic blood pressure and pulse rate of the unanesthetized  
dog after oral administration*

COMPOUND	DOSE	RESULTS							
		(Pulse rate/systolic blood pressure)							
		Time in minutes							
	mgm / kgm.	0	5	15	30	45	60	90	120
Isuprel Hydro- chloride	0.1	100/120	170/120	146/120	130/115	140/90		140/90	
	0.1	60/150	76/180	65/170	60/140	50/135			
	0.1	65/125	108/150	112/140	70/130	58/125	52/125		
	0.2	80/130	94/134	96/128	108/130		82/130		
	0.2	84/156		116/154	96/138		74/138		
	0.5	66/146	156/128	120/118	86/124	76/120	70/116	66/122	
	1.0	56/120	130/170	132/150	80/120	82/124	82/126	90/110	
O-4, 1424 Acetate	0.2	64/160	218/104*	196/128	160/148	120/150	100/150	72/154	70/158
	0.2	52/132	88/138	82/132	52/128	54/130	48/136		
	0.5	52/120	90/148		92/130		100/114		88/126
	1.0	64/136	84/154		112/118	94/124	116/118	72/114	60/136

\* Evidence of nausea; retching.

to decline to levels distinctly below those of the control period. This effect was quite prolonged (table 2). It would seem not improbable that the initial rise in systolic pressure resulted from an increased cardiac action. Results obtained with the N-sec. butyl compound were essentially the same as those obtained with Isuprel except that this latter substance seems to be somewhat more active.

EFFECT ON THE BRONCHIOLES. Isolated guinea pig lungs were perfused according to the method of Sollmann and von Oettingen, as modified by Thornton (3). Histamine acid phosphate was used to induce constriction and the rate of flow compared with that following the administration of histamine plus bronchodilator compound. The results obtained are shown in table 3. The two homologues are as effective as epinephrine in antagonizing the bronchoconstrict-

ing action of histamine. The bronchodilation induced by ten micrograms of either compound lasted for approximately twenty minutes.

The effectiveness of these substances in preventing bronchoconstriction in "experimental asthma" was determined as described below.

Guinea pigs of 250 to 450 grams weight were used as test animals. The animal was confined in a glass container of about four-liter capacity and exposed to a 0.2 per cent solution of histamine diphosphate in the form of a finely nebulized mist. The mist was produced by a standard commercial model nebulizer attached to an air line maintained at a constant pressure of 300 mm. Hg. The data were tabulated under two headings, ONSET and DURATION. ONSET represents the elapsed time in minutes and tenths, from the time the guinea pig was subjected to the histamine mist until obvious symptoms of asthma were noted. The criterion was labored breathing, with definite signs of forced inspiration. This was frequently preceded by sneezing or coughing, but since many animals sneezed almost immediately upon breathing the mist this was not taken as an indication of onset. DURATION equals the time elapsing between

TABLE 3  
Bronchodilator action  
Perfused Guinea Pig Lung Preparation

COMPOUND	RESPONSE TO HISTAMINE	RESPONSE TO HISTAMINE + 0.01 MGX COMPOUND
	cc/min	
Isuprel Hydrochloride	50/33*	53/55
O-4, 1424 Acetate	44/23	49/52
Epinephrine	50/24	48/49

\* Rate before histamine/rate after histamine

the beginning of the exposure and the time that collapse or asphyxial convulsions were observed. A six-minute period was arbitrarily chosen as the maximum time of exposure to histamine. Animals that did not show signs of toxicity within this period were considered to be completely protected. A 2-4-hour period was allowed between exposure to histamine. Both drugs were given in dilute solution and injected intraperitoneally. Fifteen minutes were permitted to elapse between drug administration and exposure to the histamine mist. Results obtained are shown in table 4. Doses as small as 0.025 mgm./kgm. of either drug caused a significant prolongation in the time of onset of asphyxial signs and diminished the severity of histamine shock.

**EFFECT ON THE INTISTINE** The effect of the two homologues on segments of guinea pig ileum was determined by the method of Magnus. A dilution of one part in ten to forty million caused a reduction in tonus and motility. Epinephrine gave comparable results in these dilutions (table 5), but concentrations as great as one part in two million caused a strong contracture rather than relaxation. At this concentration, the two homologues caused only relaxation.

This inhibitory action on the intestine can be demonstrated on the organ

Systolic blood pressure was determined in unanesthetized dogs by means of a mercury or aneroid manometer to which was attached a cuff specially designed to fit snugly about the thigh. In most dogs, a strong pulse may be obtained from the anterior tibial artery along the dorsal surface of the foot and, in these, systolic pressure may be determined by palpation. The oral administration of 0.1 to 0.5 mgm./kgm. of Isuprel caused a marked tachycardia which developed rapidly following the administration of the drug. Associated with this increase in heart rate, there was usually a rise in systolic pressure. Although pressure rose, there was evidence of peripheral vasodilatation, inasmuch as unpigmented skin areas became quite pink. As the cardiac effect of the drug diminished, blood pressure rapidly returned to normal and, in most experiments, continued

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ing action of histamine. The bronchodilation induced by ten micrograms of either compound lasted for approximately twenty minutes.

The effectiveness of these substances in preventing bronchoconstriction in "experimental asthma" was determined as described below.

Guinea pigs of 250 to 450 grams weight were used as test animals. The animal was confined in a glass container of about four-liter capacity and exposed to a 0.2 per cent solution of histamine diphosphate in the form of a finely nebulized mist. The mist was produced by a standard commercial model nebulizer attached to an air line maintained at a constant pressure of 300 mm. Hg. The data were tabulated under two headings, ONSET and DURATION. ONSET represents the elapsed time in minutes and tenths, from the time the guinea pig was subjected to the histamine mist until obvious symptoms of asthma were noted. The criterion was labored breathing, with definite signs of forced inspiration. This was frequently preceded by sneezing or coughing, but since many animals sneezed almost immediately upon breathing the mist this was not taken as an indication of onset. DURATION equals the time elapsing between

TABLE 3  
Bronchodilator action  
Perfused Guinea Pig Lung Preparation

COMPOUND	RESPONSE TO HISTAMINE	RESPONSE TO HISTAMINE + 0.01 MCM. COMPOUND
	cc./min	
Isuprel Hydrochloride . . .	50/33*	53/55
O-4, 1424 Acetate . . .	44/23	49/52
Epinephrine . . . . .	50/24	48/49

\* Rate before histamine/rate after histamine.

the beginning of the exposure and the time that collapse or asphyxial convulsions were observed. A six-minute period was arbitrarily chosen as the maximum time of exposure to histamine. Animals that did not show signs of toxicity within this period were considered to be completely protected. A 2-4-hour period was allowed between exposure to histamine. Both drugs were given in dilute solution and injected intraperitoneally. Fifteen minutes were permitted to elapse between drug administration and exposure to the histamine mist. Results obtained are shown in table 4. Doses as small as 0.025 mgm./kgm. of either drug caused a significant prolongation in the time of onset of asphyxial signs and diminished the severity of histamine shock.

EFFECT ON THE INTESTINE. The effect of the two homologues on segments of guinea pig ileum was determined by the method of Magnus. A dilution of one part in ten to forty million caused a reduction in tonus and motility. Epinephrine gave comparable results in these dilutions (table 5), but concentrations as great as one part in two million caused a strong contracture rather than relaxation. At this concentration, the two homologues caused only relaxation.

This inhibitory action on the intestine can be demonstrated on the organ



TABLE 4

*Protective action in guinea pigs exposed to histamine mist*

COMPOUND	DOSE	RESULTS (IN MINUTES)				NO OF EXPERIMENTS
		Before Medi- cation		After Medication		
		Onset†	Dura- tion‡	Onset	Duration*	
Isuprel Hydro- chloride	<i>mgm /kgm</i> 0.025	0.88	1.43	2.78	3.66	5
	0.100	0.76	1.52	3.06	>6.00 in six experi- ments	12
	0.250	1.35	2.76	>6.00		
0-4, 1424 Acetate	0.025	0.84	1.30	2.66	3.74	10

\* A six minute interval was arbitrarily chosen as the maximum time of exposure to histamine. Animals not showing symptoms within this period were considered to be completely protected.

† Elapsed time between the beginning of exposure and the onset of respiratory difficulty.

‡ Elapsed time between the beginning of exposure and the onset of collapse or asphyxial convulsions.

TABLE 5

*Effect on the intestine and uterus***Organs Excised**

COMPOUND	ILEUM		UTERUS†			
	Guinea Pig		Rabbit		Guinea Pig	
	Dilution	Effect	Dilution	Effect	Dilution	Effect
Isuprel Hydrochloride	50M (million) 100M	I*	20M-40M	I	4M-10M	I
0-4, 1424 Acetate	10M-20M	I	10M-20M	I	4M-10M	I
Epinephrine	10M-40M	I	10M	E*	4M	E

**Organs in situ**

COMPOUND	DOSE	RABBIT ILEUM	RABBIT COLON	RABBIT UTERUS†	
		Duration of inhibition	Duration of inhibition	Effect	Duration
	<i>mgm /kgm</i>	<i>minutes</i>	<i>minutes</i>		<i>minutes</i>
Isuprel Hydrochloride	0.25	28	17	I	17->20
	0.11				
	0.50				
0-4, 1424 Acetate	0.50	10-15	10	I	8-16
	0.10				
	0.50				
Epinephrine	0.01-0.10			E	3-6

\* I = inhibits, E = excites

† Non gravid uteri were used in all instances

*in situ*. Motility and tonus of the rabbit small intestine and colon were recorded by the method described by Jackson (4a). Doses of 0.05 to 0.30 mgm./kgm. caused a prompt reduction in tonus and motility (fig. 3 and table 5). The results obtained suggest that the N-sec. butyl derivative is slightly less effective than Isuprel.

**EFFECT ON THE UTERUS.** The effect of these substances on the isolated non-gravid uterus of the rabbit and guinea pig was determined as described above for the intestine. Pitressin was added to the bath solution to induce activity when the segment did not contract spontaneously. The results obtained are shown in table 5. Both derivatives caused inhibition of motility in high dilution in both animal species. By comparison, epinephrine in these dilutions caused

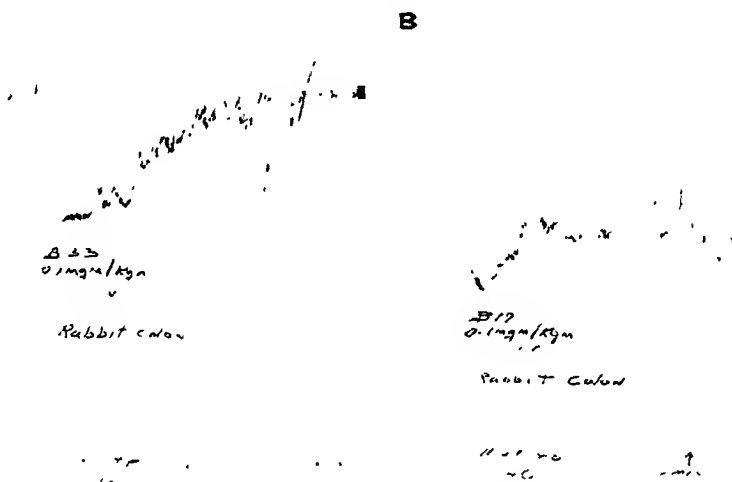


FIG. 3. EFFECT ON THE RABBIT COLON IN SITU

A. N-sec. butyl derivative (B 33) in an intravenous dose of 0.1 mgm./kgm.

B. Isuprel HCl (B 17) in an intravenous dose of 0.1 mgm./kgm.

Time intervals in minutes.

stimulation. Rabbits treated with diethylstilbestrol, 3 micrograms/kgm. every second day for a total of 6 injections, were prepared in the manner described by Jackson (4b) for the recording of the motility of the uterus, *in situ*. Both homologues, in intravenous doses of 0.5 mgm./kgm., caused strong inhibition of uterine motility (table 5). By comparison, epinephrine, in intravenous doses of 0.01 to 0.10 mgm./kgm., caused strong tonic contraction of this organ.

**TOXICITY.** Acute toxicity was determined in albino mice by intraperitoneal injection. All mice were from our own colony, weighed 15-21 grams, and both before and during the test were housed at a constant temperature of 76°F. Deaths occurring within 72 hours were recorded. Examination of table 1 shows that the two derivatives are of comparable toxicity and that both are much less toxic than epinephrine.

TABLE 4  
Protective action in guinea pigs exposed to histamine mist

COMPOUND	DOSE	RESULTS (IN MINUTES)				NO OF EXPERIMENTS
		Before Medication		After Medication		
		Onset†	Duration‡	Onset	Duration*	
	mgm /kgm					
Isuprel Hydrochloride	0.025	0 88	1.43	2.78	3.66	5
	0 100	0 76	1 52	3.06	>6.00 in six experiments	12
	0.250	1 35	2 76	>6 00		
0-4, 1424 Acetate	0 025	0 84	1.30	2.66	3 74	10

\* A six minute interval was arbitrarily chosen as the maximum time of exposure to histamine. Animals not showing symptoms within this period were considered to be completely protected.

† Elapsed time between the beginning of exposure and the onset of respiratory difficulty.

‡ Elapsed time between the beginning of exposure and the onset of collapse or asphyxial convulsions.

TABLE 5  
Effect on the intestine and uterus

Organs Excised

COMPOUND	ILEUM		UTERUS†			
	Guinea Pig		Rabbit		Guinea Pig	
	Dilution,	Effect	Dilution,	Effect	Dilution	Effect
Isuprel Hydrochloride	50M (million) 100M	I*	20M-40M	I	4M-10M	I
0-4, 1424 Acetate	10M-20M	I	10M-20M	I	4M-10M	I
Epinephrine	10M-40M	I	10M	E*	4M	E

Organs in situ

COMPOUND	DOSE 11	RABBIT ILEUM	RABBIT COLON	RABBIT UTERUS†	
		Duration of inhibition	Duration of inhibition	Effect	Duration
	<i>mgm /kgm</i>	<i>minutes</i>	<i>minutes</i>		<i>minutes</i>
Isuprel Hydrochloride	0 25	28			
	0 11		17		
	0 50			I	17->20
0-4, 1424 Acetate	0 50	10-15			
	0 10		10		
	0 50			I	8--16
Epinephrine	0.01-0.10			E	3--6

\* I = inhibits, E = excites

† Non-gravid uteri were used in all instances.

## SUMMARY

1. The replacement of the N-methyl group of epinephrine by -isopropyl or -sec. butyl groups results in compounds that are strongly depressor. This depressor action is comparable to that sometimes elicitable with epinephrine.
2. The homologues of epinephrine have a direct stimulating effect on the heart.
3. The N-isopropyl and -sec. butyl homologues of epinephrine dilate the bronchioles, relax the small intestine, colon, and uterus, when present in high dilution.
4. The N-isopropyl homologue appears to be somewhat more effective than the N-sec. butyl homologue.
5. The possible role of these agents as sympathin I-mimetic agents is discussed.

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The effects of daily oral administration of Isuprel were determined. This drug was incorporated into a standard rat diet to make 0.5 per cent by weight, and this mixture was given as the only source of food to 20 weanling rats for 7 weeks. There was no significant difference between the rate of growth of these rats and that of a comparable control group. A complete pathological examination was made. No changes suggesting toxic effects of the drug were noted.

**DISCUSSION.** The fall in blood pressure sometimes resulting from the intravenous injection of small amounts of epinephrine is a well-known phenomenon. The investigations of Cannon and Lyman (5), Hartmann (6), and Hartmann and Kilborn (7) have shown this effect to result from peripheral vasodilatation. Konzett (1b) has recently reported that vasodilatation is prominent with the N-propyl, -isopropyl, -butyl, and -isobutyl homologues of epinephrine. This investigator has found that the N-isopropyl homologue dilates the coronary vessels; is an effective bronchodilator; relaxes the isolated rabbit jejunum; and is without effect on the gravid uteri of rabbits or on the denervated nictitating membrane of cats. Such effects might be expected to result from a suppression of the excitatory (sympathin E) effects of epinephrine or to result from a modification of the molecular structure of epinephrine so that specific stimulation of the depressor mechanism results. Lands, Rickards, Nash and Hooper (2) have reported that depressor effects are prominent with the homologues of 1-(p-hydroxyphenyl)-2-aminoethanol when one of the hydrogens on the nitrogen has been replaced by ethyl, isopropyl, secondary butyl or tertiary butyl groups; but not when replaced by propyl, butyl or isobutyl groups. It was further shown that the removal of the beta-hydroxyl group from the side chain led to a loss of the specific effects described. This suggests that there are definite structural requirements for depressor effects comparable to those that have been described as essential for pressor effects. The results obtained in the experiments reported in this communication have shown that the secondary butyl homologue is qualitatively equivalent but slightly less potent than the isopropyl homologue. Both of the above substances closely resemble their 1-(p-hydroxyphenyl)-2-aminoethanol analogues. Cannon and Rosenblueth (8) have postulated that sympathetic inhibition leads to the liberation into the blood stream of a substance capable of causing inhibitory effects on other sympathetically innervated structures similarly affected by epinephrine. In that sense, the N-isopropyl and N-sec. butyl homologues of epinephrine described here may be designated as sympathin I-mimetic agents. When present in the blood stream in high dilution, they lower blood pressure, dilate the bronchioles, relax the uterus, small intestine and colon, and apparently do not cause contraction of the denervated nictitating membrane (N-isopropyl homologue described by Konzett, 1b). However, the stimulating effect on the heart would seem to be an exception to this generalization. Cannon and Bacq (9) have shown that sympathetic discharge into the uterus and bladder will cause acceleration of the denervated heart. It is not improbable that this acceleration resulted from the liberation of excitatory (sympathin E) rather than inhibitory (sympathin I) substances. The cardiac effects of sympathin I appear not to have been determined.

a suitable time before the protein. A translation of this paper has not been available to us (although we did get the conclusions translated) and we do not know the details of his work. Lavollay and Neumann (12) stated that in the anesthetized dog quercitrin protected the animal from the profound drop in blood pressure observed in peptone shock. The present report is concerned with those of our studies on rutin related to the papers mentioned above.

**EXPERIMENTAL** *Action of rutin on excised intestine* Segments of guinea pig colon were suspended in 50 ml of aerated Ringer-Locke solution maintained at a temperature of 38°C. Epinephrine hydrochloride was added to the bath in such amount as to cause clear but not maximal relaxation, and the rutin solution to be tested had a concentration of 1 mgm per ml. The rutin<sup>1</sup> was dissolved in hot water from which it would precipitate gradually on cooling.

The protective action of rutin on epinephrine as observed by Lavollay was verified. 0.2 mgm of rutin in 50 ml of bath always prolonged the action of

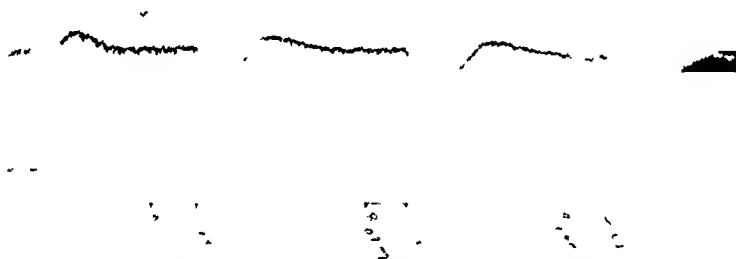


FIG. 1. THE PROLONGED ACTION OF 10 GAMMA OF EPINEPHRINE IN THE PRESENCE OF RUTIN

E = epinephrine, R = rutin. The amounts of rutin in the 50 ml of bath were 0.2, 0.4 (0.2 + 0.2), and 1.0 (0.2 + 0.2 + 0.6) mgm. The rutin had no direct effect on the muscle strip.

the epinephrine, lower concentrations failed to do so. The time of prolongation was found to be directly proportional to the dosage of rutin (fig. 1). Ordinarily the degree of relaxation due to epinephrine was not modified by the presence of rutin although occasionally the relaxation was augmented. A single dose of rutin, if left in the bath, was effective in prolonging the action of several doses of epinephrine.

Lavollay (14) has stated that the flavonols which he studied had no direct action on the guinea pig colon. With the lower of those concentrations which would protect the action of epinephrine we confirmed his statement. However, when the concentration of rutin was increased there was sometimes a direct action of the rutin on the intestinal strip (fig. 2). Addition of 0.5 mgm. of rutin to 50 ml of bath caused infrequent relaxation, 1 mgm per 50 ml led to relaxation about once in every three times and higher concentrations about half of the time.

<sup>1</sup>The rutin was furnished us by the Eastern Regional Research Laboratory of the U. S. Department of Agriculture, Philadelphia.

## SOME PHARMACOLOGICAL PROPERTIES OF RUTIN

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Rusznayák and Szent-Györgyi (1) announced in 1936 that they had found indications of a nutritional factor which influenced capillary permeability. They called it vitamin P ("P" for permeability). Zacho (2) was able to produce capillary fragility in guinea pigs and then cure it with vitamin P and Rusznayák and Benkó (3) did the same with rats. However, others were not as successful, and evidence for the factor remained uncertain (4). Scarborough (5) obtained clinical results supporting the existence of the vitamin. Bacharach, Coates, and Middleton (6) stated that they could utilize the prevention of capillary fragility of guinea pigs as an assay procedure. Recently, following the paper of Griffith, Couch and Lindauer (7), there have been a number of favorable reports (5, 8, 9) from clinicians on the efficacy of rutin in patients with increased capillary fragility. All preparations so far tested which have a vitamin P activity have been found to be related to the flavonols, and of them, rutin (quercetin rhamnoglucoside) can be prepared readily in pure form (10). While clinical evidence accumulates that some such factor exists, experimental evidence is scanty. Positive results have been indicated above. Others have been unable to confirm their findings, although published reports of such negative results are few. This paper is not an argument for or against the existence of vitamin P, but we have used the term because it is convenient and is established in the literature.

Reports of pharmacological studies on vitamin P are few, largely, perhaps, because the substances supposed to have vitamin P activity produce no striking physiological actions. Wilson, Mortarotti, and Dostader (11), for example, found no acute or chronic toxicity with large doses of rutin. The papers to be reviewed here are those which apply most directly to the work to be reported. The most extensive pharmacological studies come from a French group (12, 13) investigating the activity on isolated organs of the guinea pig of a number of flavonols and related compounds. They concluded that these derivatives had no direct action on the isolated intestine (Lavollay, (14)), but that by means of their antioxidant properties, they protected epinephrine from destruction so that its effect was prolonged (Lavollay (15)). Lavollay and Neumann (12) reported that quercitrin would prolong the action of epinephrine on blood pressure in the dog. Parrot (16) could not confirm this, although with epicatechin (a substance related to the flavonols), he obtained a prolonged epinephrine contraction of the nictitating membrane of the cat.

Hiramatsu (17) gave "vitamin P (hesperidin)" to guinea pigs and found that there was protection from anaphylactic shock if the substance were given

a dose was determined to be 0.25 mgm of histamine base per kilogram of body weight. The injections were ordinarily into the jugular vein after the vein had been exposed following procaine anesthesia. Occasionally injections were made into the femoral vein, also with procaine anesthesia.

The results of this experiment are tabulated in Table 1. It was noticed that considerable protection against histamine shock was obtained when rutin had preceded the histamine by certain intervals. An injection of rutin 10-30 minutes before the histamine considerably increased the percentage of surviving animals, but with longer intervals between the two drugs the mortality was as great as that of the controls. That the protection was not instantaneous and

TABLE 1  
*Effect of rutin on histamine shock*

	RUTIN	HISTAMINE	NUMBER OF ANIMALS USED	NUMBER SURVIVING	PER CENT SURVIVING
	mgm	mgm/kg			
Controls					
Rutin, no histamine	5 i v	0	2	2	100
	10 i v	0	3	3	100
Histamine, no rutin	0	0.25	24	10	42
Experimental					
Time after Rutin, min					
0	5 i v	0.25	8	1	13
9-15	10	0.25	8	7	87
20-21	10	0.25	6	5	83
25-35	10	0.25	3	3	100
36-45	10	0.25	6	3	50
56-65	10	0.25	11	6	55
Rutin feeding	10 mgm / day	0.25	4	2	50

Rutin (1 per cent solution in 5 per cent ethyl alcohol) given intraperitoneally, 1 ml per animal, except where otherwise noted. Histamine injections were intravenous.

presumably was not a direct antagonism between rutin and histamine was shown when 5 mgm of rutin solution was mixed with the appropriate dose of histamine and injected intravenously. A smaller quantity of rutin was used in this case to keep the volume of the intravenous injection as small as possible, even so, the concentration of rutin in the blood stream must have been higher than occurred when 10 mgm were injected intraperitoneally. There was no protection of the animals when this routine was practiced. The deaths of the animals cannot be attributed to the rutin itself because 5 or 10 mgm of rutin injected intravenously did not embarrass the animals in any degree. Nor was the protection due to the alcohol in the solution as 1 ml. of 5 per cent ethyl alcohol 20-30 minutes before the histamine did not modify the mortality.



Quercetin, the aglycone of rutin, was much more effective and constant in causing relaxation of intestinal strips and in prolonging the relaxation produced by epinephrine. The actions on intestinal strips of histamine and acetylcholine were not modified by rutin.

*Action of rutin on histamine shock.* Hiramatsu (17) has reported on the protection of guinea pigs from anaphylactic shock by pre-treatment with vitamin P. In a preliminary study, which is not being reported in detail because the data are too meager, we were unable to confirm this finding. However, the following experiment demonstrated that rutin would protect against histamine shock under specific conditions.

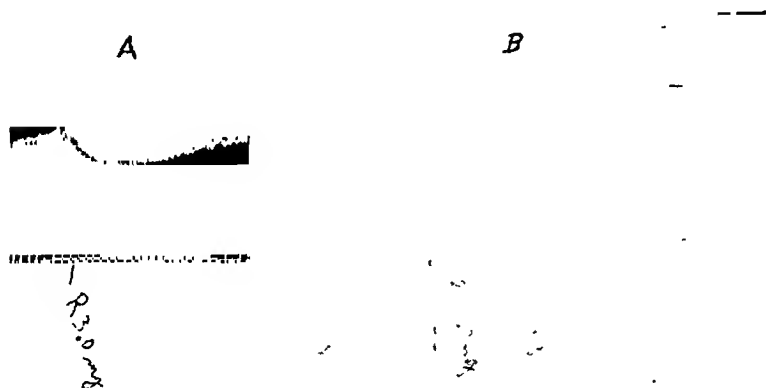


FIG. 2. DIRECT ACTION OF RUTIN ON THE INTESTINAL STRIP

In A, 3 mgm. of rutin caused a prolonged relaxation with little action on peristalsis. In B, 2 mgm. of rutin caused relaxation, and a further and greatly prolonged relaxation occurred when epinephrine was added. Time in minutes.

Guinea pigs were used, 73 per cent of them weighing between 200 and 350 grams; those outside these limits reacted in the same manner as the majority. Both sexes were represented with no difference in reaction between them. The guinea pigs were placed on a scorbutic diet because such a diet was comparatively free of flavone derivatives<sup>2</sup> and would lead to more easily controlled conditions. Five mgm. supplements of ascorbic acid were given each animal four times weekly and a 10 mgm. supplement on Friday. After remaining on this diet for one or two weeks the animals were considered ready to use.

A one per cent solution of rutin in 5 per cent ethyl alcohol was injected intraperitoneally. Such a solution is readily prepared by heating and can be injected immediately after cooling, before crystallization again occurs. The dosage was ordinarily 1 ml. (10 mgm.) per animal. Histamine dihydrochloride was injected intravenously in such amount as to kill, by bronchial constriction, about half of the animals; under the conditions of this experiment such

<sup>2</sup>The diet consisted of dried skim milk (28 parts), brewer's yeast (4 parts), rolled oats (24 parts), wheat bran (42 parts), and cod liver oil (2 parts). To examine for the presence of flavonol derivatives, the various constituents of this diet were extracted with ethyl alcohol in a Soxhlet extractor for twenty-four hours and the dried extracts were tested by means of the boric acid color reaction (18) and by the cyanidin method (19). Both methods indicated negligible quantities of flavonol derivatives.

The guinea pigs were 300 gram albinos placed on the scorbutic diet mentioned earlier, or on the same diet modified by the elimination of the yeast. Supplements, where used, were given daily, Monday through Friday (with a double dose on Friday) by pipette—5 mgm. of ascorbic acid for prevention or cure of scurvy and 10 mgm. of rutin (1 per cent solution in 5 per cent ethyl alcohol) for the possible prevention or cure of capillary fragility. The negative pressure method was used for determining capillary fragility—the measurements usually being made on Friday so that the varied schedule over the week-end would have a minimal effect. Essentially, the method as described by Bacharach, Coates, and Middleton (6) was employed, although they cautioned against the use of a depilatory, while we found that there was less skin trauma when using a depilatory than after shaving.

The following three groups of animals were studied: (1) ascorbic acid was administered to all and rutin to half of the animals in this group. No scurvy developed in any of these animals. (2) Scurvy was developed in all animals with subsequent cure—half of the guinea pigs receiving rutin from the beginning of the experiment. (3) The same as group (2) except that the rutin supplements began after scurvy had developed. We could discover no correlation between the negative pressure required to produce petechial hemorrhage and the presence or absence of rutin. There was little correlation between the results of the two observers, and indeed, there was sometimes a marked discrepancy in the two readings made by a single observer on adjacent areas of skin of a single animal.

**DISCUSSION.** The protection of epinephrine by flavonols in isolated organ experiments, as demonstrated by the French workers, has been confirmed. The protection is proportional to the dose of rutin, and the rutin is effective for a considerable time if allowed to remain in contact with the muscle strips. There appears to be no toxic action on the isolated intestine. Unlike the French group we found that with increased dosage of rutin and with quercetin that a direct action on the muscle strip was sometimes obtained. This action consisted of a decrease in tonus and an epinephrine relaxation could be superimposed unless the initial direct reaction was extreme.

Protection of guinea pigs from histamine shock by preliminary treatment with rutin is clear but not spectacular. The conditions which favor this protection are quite specific in that too short or too long a time between the two substances leads to no protection against death. It is possible that concentration of the rutin would be a factor also, although in these experiments variation in the dosage of rutin was not studied. Our inability to confirm Hiramatsu's conclusions on the protective action of vitamin P in anaphylactic shock may well be due to differences in experimental procedures between his work and ours. Details of his work are not available to us, and we used too few animals in anaphylactic studies to check his conclusions adequately.

This protection against asphyxial death from histamine is not a direct antagonism of histamine by rutin but is mediated in some indirect manner. Using isolated organs (lung or intestine), little or no modification of histamine contraction was obtained by the simultaneous administration of rutin. The mode of action of the rutin in protecting against histamine shock cannot be stated at this time, but a tentative suggestion can be made. The intestinal strips clearly indicated that rutin protects epinephrine from rapid destruction.

One group of four animals on the scorbutic diet was fed rutin by pipette, 10 mgm. per day. One day after the last rutin supplement, these animals were injected intravenously with histamine, without protection.

*Action of rutin and histamine on isolated organs.* In the preceding section it was shown that under certain conditions rutin would protect guinea pigs from asphyxial death due to histamine. However, it was indicated that the action was probably not a direct one between the rutin and the histamine. To study this further, isolated guinea pig lungs were used for bronchiolar perfusion. The technic of Thornton (20) as modified by Ambrose and DeEds (21) was used and the perfusing solution was that of van Dyke and Hastings (22), either alone or containing 3 mgm. per-cent of rutin. In all four experiments performed in this way, it was found that the amount of constriction due to histamine was as

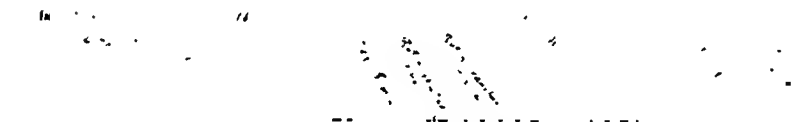


FIG. 3. BRONCHIAL PERFUSION OF ISOLATED GUINEA PIG LUNGS

Histamine caused constriction, with a resultant rise in the pressure of the system. In the presence of 3 mgm. of rutin in each 100 ml. of perfusate, the constriction with histamine was as great as before.

great when the perfusate contained rutin as it was in its absence (fig. 3). Using strips of guinea pig colon, it appeared that constriction from histamine was about as great in the presence of rutin as in its absence. These are further indications that the action of rutin in histamine shock was not direct.

*Capillary fragility in the guinea pig.* In the introduction the results of Bacharach, Coates, and Middleton (6) were cited showing that in their hands it was possible to use the capillary fragility of guinea pigs to assay vitamin P activity. Lavollay and Sevestre (23) have used guinea pigs successfully to determine vitamin P activity, but they have not been able to produce capillary fragility under the conditions of Bacharach's experiment, where the animals are protected from scurvy by ascorbic acid. Other workers, mostly by word of mouth, have indicated that protection of experimental animals against capillary damage was frequently not successful. For the benefit of the record, our negative results are included as a possible aid to others trying to use this method.

on guinea pigs was not effective. A modification of this method, or some other method that would work in all laboratories, is highly desirable.

Following the completion of this paper our attention has been called to the report of J. L. Parrot and G. Richet in the *Compt. Rend. Soc. Biol.*, **139**: 1072-1075, 1945. These authors demonstrated an increased sensitivity to histamine in scorbutic guinea pigs and reported that this increase is decreased by vitamin P administration.

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If the epinephrine which is being liberated in a normal manner by the animal is protected by rutin, the epinephrine concentration in the blood should rise slightly due to decreased destruction. Introduced histamine would then have less effect on the animal. When rutin is given simultaneously with the histamine, histamine action occurs before an increased blood concentration of epinephrine can be achieved. When the period between introduction of rutin and histamine is prolonged, it is conceivable that the rutin has been destroyed or excreted, in which case the epinephrine level of the blood would not be above normal levels.

The increase in blood epinephrine cannot be great. This is shown by the fact that protection of the animals from histamine shock was not spectacular, but required a number of animals to make the results significant. We have some preliminary unpublished experiments suggesting that, sometimes, there is an increased epinephrine content in the serum of guinea pigs treated with rutin, but because of the unsatisfactory nature of blood-epinephrine determinations, and the apparently slight amount of the epinephrine rise, a clear-cut demonstration of such a change has not yet been achieved. This work will be reported in more detail later.

A method of assay of vitamin P activity is a highly desirable and, in fact, necessary tool. It is unfortunate that the measurement of capillary fragility in experimental animals, a method which works so satisfactorily in some laboratories, cannot be applied to all in certain others. There is some condition which is not apparent which must be unconsciously observed by some of the workers and not by others, and until this condition is realized and published, the capillary fragility method will be unsuitable for assaying vitamin P potency.

#### CONCLUSIONS AND SUMMARY

1. Rutin prolongs the action of epinephrine on intestinal strips. The prolongation is presumably due to a protection of the epinephrine from oxidative destruction.

2. In somewhat larger concentrations, rutin sometimes has a direct relaxing action on the intestinal muscle.

3. Administration of rutin intraperitoneally to guinea pigs decreases the mortality due to an approximately  $LD_{50}$  of histamine, if the rutin precedes the histamine by 10 to 30 minutes. Simultaneous introduction of the two substances does not decrease the mortality, nor is there protection of the animal if the time between the two drugs is as much as 45 minutes to an hour.

4. That the protection against histamine is indirect is shown by the absence of protection when the two drugs are injected simultaneously, and by lack of antagonism on perfused isolated bronchi and on strips of guinea pig colon.

5. It is tentatively suggested that the protection of the animals from histamine shock is due to a slightly increased epinephrine level in the blood. Rutin, by an *in vivo* retardation of epinephrine destruction, would lead to an increase in epinephrine concentration.

6. In our hands, the capillary fragility method for assaying vitamin P activity

tion. The writing point consisted of a very light swinging pendulum thus allowing for very little friction. In the experiments in which electrical stimulation was used the stimuli consisted of brief shocks from a square-wave generator. The electrodes consisted of a platinum wire immersed in the fluid of the cannula and a cotton wick tipped copper wire in contact with the surface of the heart. The salt solution (Clark) employed had the following composition, sodium chloride, 0.65 per cent, potassium chloride 0.014 per cent, calcium chloride (anhydrous) 0.012 per cent, sodium bicarbonate, 0.03 per cent. The pH was about 7.6.

Iodosobenzoic acid and para-chloromercuric benzoic acid were used as their sodium salts. Solutions of all reagents were prepared immediately before the experiments and their hydrogen-ion concentration was adjusted to that of the normal Clark's solution and was kept between pH 7.5 and 7.8. All the experiments were conducted at room temperature between 23 and 25°C.

**RESULTS.** I. *The effect of oxidizing agents.* (a) *Porphyryndin.* In concentrations of  $1 \times 10^{-3}$  to  $2 \times 10^{-3}$  ( $3.7$  to  $7.5 \times 10^{-3}$  M) porphyryndin caused an immediate and gradually progressing systolic effect which terminated in systolic

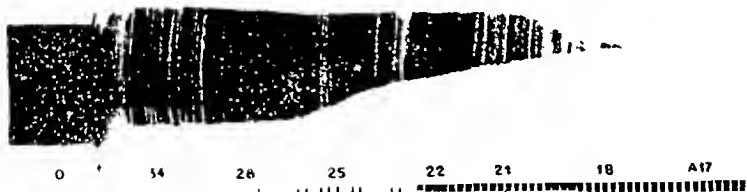


FIG. 1. THE ACTION OF PORPHYRYNDIN UPON THE ISOLATED FROG HEART  
At arrow, porphyryndin  $2 \times 10^{-3}$ . The numbers indicate heart rate per minute. A, auricle. Time in 30-second intervals.

standstill in a period of approximately 30 minutes (see fig. 1). Occasional extrasystoles but no auriculo-ventricular block were observed during this period. The auricles continued beating after the ventricle had stopped. The effect of the above concentrations was not reversible after repeated washings with Clark's solution.

Concentrations of porphyryndin of  $1 \times 10^{-4}$  to  $2 \times 10^{-4}$  produced a pronounced increase in the amplitude of contraction which gradually disappeared as the solution of porphyryndin in the cannula was losing its strong violet color. This change in color was due to the transformation of porphyryndin to its leucobase by a process of hydrogenation. In one experiment in which porphyryndin,  $2 \times 10^{-4}$ , was used the increased amplitude of contraction lasted for 90 minutes. At the end of this time the action of porphyryndin was no longer evident since the solution was almost colorless.

As the concentration necessary to cause complete systolic effect was often as high as  $2 \times 10^{-3}$  no attempt was made to study the relation between concentra-

# THE ACTION OF SULFHYDRYL REAGENTS UPON THE ISOLATED FROG HEART<sup>1</sup>

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The discovery of the presence of electronegative sulfhydryl groups in a large variety of enzymes has been a subject of considerable attention in the last few years. Sulfhydryl groups have been demonstrated among certain hydrolytic, proteolytic and oxidation enzymes. Recently Barron and Singer (1, 2) have demonstrated that their presence is essential for the activity of a considerable number of enzymes concerned with the metabolism of carbohydrates, fats and proteins. For a recent review of the subject see (1, 2).

The achievement of systolic standstill on the isolated frog heart by specific reagents which combine with the sulfhydryl groups of the sulfhydryl-containing enzymes has been described recently by one of us in a preliminary note (3). The fact that the systolic effect caused by the peroxide-forming alpha-beta and beta-gamma angelicalactones (4) could be prevented by cystine and glutathione lead to the idea that the effect caused by certain peroxides upon the isolated frog heart could be mediated by an oxidation process which would interfere with the normal functions of the sulfhydryl enzymes.

In the present paper we report in detail the action of certain specific sulfhydryl reagents upon the frog heart and the effect of the addition of free-SH groups in preventing and reversing the effects of such reagents. The sulfhydryl reagents selected by Barron and Singer, and by Hellerman, Chinard and Dietz (5) as the most specific and reliable have been used by us in the experiments described below. These reagents are: two oxidizing agents, porphyrindin and sodium ortho-iodosobenzoate; an alkylating reagent, iodoacetamide; two mercaptide-forming compounds, sodium para-chloromercuric benzoate and 3-amino, 4-hydroxy-benzene-arsenous hydrochloride.

Porphyrindin is an oxidation-reduction dye, a derivative of diazine, which is not commercially available. The porphyrindin as well as the iodoacetamide used in this study were prepared by Dr. E. S. Guzman-Barron. Iodosobenzoic acid and glutathione were obtained from Eastman Kodak. Para-chloromercuric benzoic acid was prepared by Dr. Leslie Hellerman, and the arsenical compound was generously supplied by Parke Davis and Company.

**METHODS.** The experiments were carried out throughout the year on male frogs (*Rana Pipiens*) weighing from 30 to 40 gm. The Straub-Fühner technique was used. Preparations which leaked even very slightly were promptly discarded. The inscripitory lever was balanced with plastiline for optimal weight compatible with satisfactory inscrip-

<sup>1</sup> Part of the expenses of this work were defrayed by a grant to one of us (Rafael Mendez) from the Ella Sachs Plotz Foundation.

veloped, however, after a period of several minutes. During this period the ventricle was apparently stopped, but no electrogram was taken to ascertain whether there was a travelling wave of excitation. The systolic effect of iodoacetamide was accompanied by an increase in heart rate.

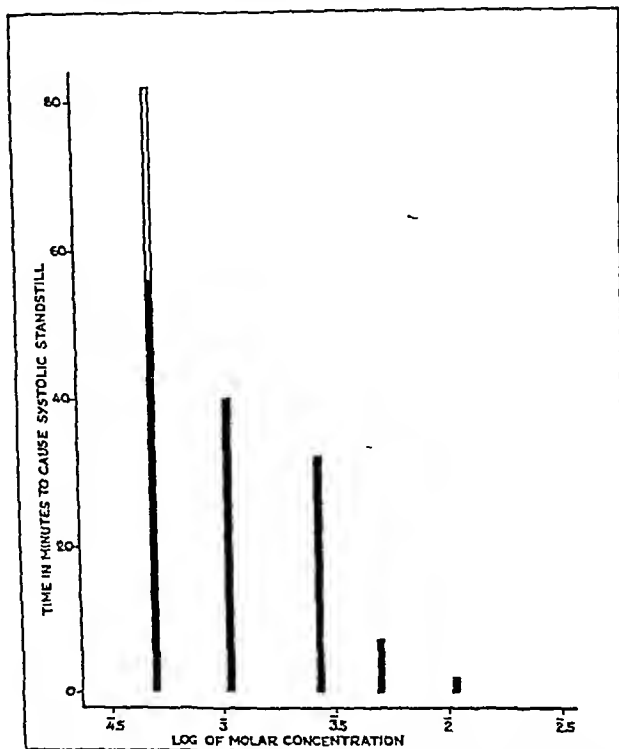


FIG 3 IODOACETAMIDE

Relation between molar concentration of iodoacetamide and time necessary to cause systolic standstill. The hollow portion of the first column on the left represents the delay in the production of systolic standstill when iodoacetamide was replaced by glutathione 25 minutes after application of the former (2 experiments)

III *The effect of the mercaptide-forming compounds.* (a) *Sodium-para-chloromercuric benzoate*. The study of the mercaptide-forming compounds was not as simple as that of the oxidizing agents and iodoacetamide. Complete systolic effect was obtained only when high concentrations were used. Low concentrations caused a predominantly diastolic effect.

A concentration of  $2 \times 10^{-5}$  ( $5.6 \times 10^{-5}$  M) of para-chloromercuric benzoate did not produce consistent results. Figure 5 represents the average type of result obtained with this concentration. In this experiment para-chloromercuric benzoate caused a purely depressant effect with normal rhythm and



tion and time taken to cause systolic standstill. All the concentrations of porphyrindin studied caused a decrease in heart rate.

(b) *Sodium ortho-iodosobenzoate*. A concentration of  $2 \times 10^{-4}$  ( $8.3 \times 10^{-4} M$ ) caused an increase in the amplitude of contraction followed by a systolic effect. Systolic standstill was brought about in a period of 20 to 30 minutes (fig 2). Occasional extrasystoles were observed in some experiments. As a rule no auriculo-ventricular block was observed before the ventricle stopped in complete systole. A concentration of  $1 \times 10^{-3}$  caused systolic standstill in a period of approximately 10 minutes. The systolic arrest caused by iodosobenzoate could not be reversed after repeated washings with Clark's solution. As in the case of porphyrindin concentrations of iodosobenzoate smaller than those necessary to produce systolic standstill ( $1 \times 10^{-4}$  to  $2 \times 10^{-5}$ ) caused a temporary increase in the amplitude of contraction.

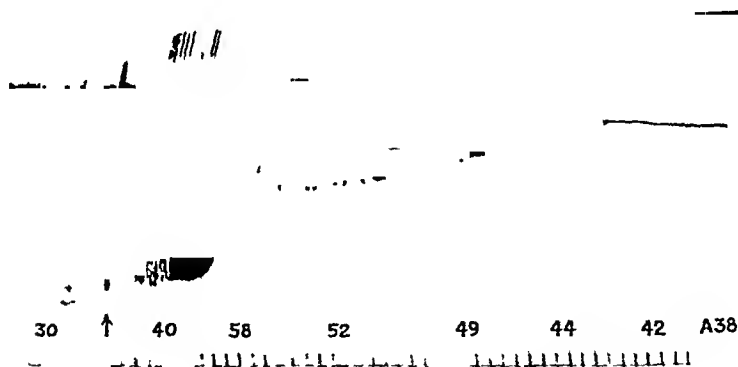


FIG 2 THE ACTION OF SODIUM ORTHO IODOSOBENZOATE ON THE ISOLATED FROG HEART  
At arrow, sodium ortho iodosobenzoate  $2 \times 10^{-4}$ . The numbers indicate heart rate per minute. A, auricle. Time in 30 second intervals.

All the above concentrations of iodosobenzoate caused an increase in heart rate. When concentrations which produce systolic standstill were used, the heart rate dropped as the ventricle was approaching the final stage of the action of the compound (see fig 2).

II *The effect of the alkylating reagent, iodoacetamide*. Systolic standstill of the frog ventricle can be obtained with iodoacetamide in concentrations ranging from  $1 \times 10^{-4}$  to  $2 \times 10^{-3}$  ( $5.4 \times 10^{-4}$  to  $1.08 \times 10^{-2} M$ ). The relation between concentration and time required to cause complete systolic effect is expressed in figure 3. Each value represents the average of 5 experiments. Concentrations of  $2 \times 10^{-3}$  to  $5 \times 10^{-4}$  caused a slight depressant effect of about one minute duration followed by an increased amplitude of contraction and a systolic effect which terminated in ventricular contracture (fig 4). Concentrations of  $2 \times 10^{-4}$  to  $1 \times 10^{-4}$  caused an increase in amplitude of contraction which terminated in ventricular arrest in half or one-third systole. Ventricular contracture de-

veloped, however, after a period of several minutes. During this period the ventricle was apparently stopped, but no electrogram was taken to ascertain whether there was a travelling wave of excitation. The systolic effect of iodoacetamide was accompanied by an increase in heart rate.

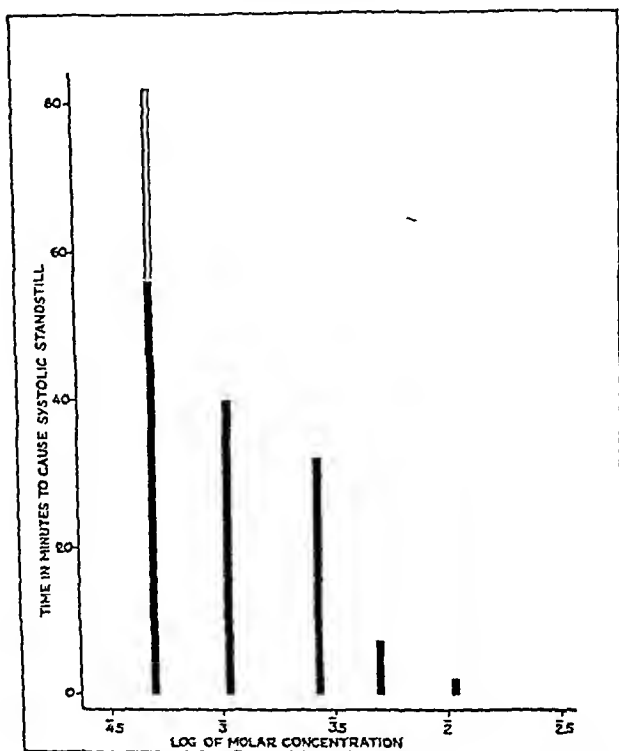


FIG 3 IODOACETAMIDE

Relation between molar concentration of iodoacetamide and time necessary to cause systolic standstill. The hollow portion of the first column on the left represents the delay in the production of systolic standstill when iodoacetamide was replaced by glutathione 25 minutes after application of the former (2 experiments)

III The effect of the mercaptide-forming compounds (a) *Sodium-para-chloromercuric benzoate*. The study of the mercaptide-forming compounds was not as simple as that of the oxidizing agents and iodoacetamide. Complete systolic effect was obtained only when high concentrations were used. Low concentrations caused a predominantly diastolic effect.

A concentration of  $2 \times 10^{-5}$  ( $5.6 \times 10^{-5}$  M) of para-chloromercuric benzoate did not produce consistent results. Figure 5 represents the average type of result obtained with this concentration. In this experiment para-chloromercuric benzoate caused a purely depressant effect with normal rhythm and

decreased amplitude of contraction lasting for 2 to 3 minutes. This was followed by a gradually developing systolic effect with auriculo-ventricular dissociation. The systolic effect which brought the ventricle to a stage of approximately two-thirds systole lasted for 15 minutes, but gradually reverted to the initial stage of depression with normal relaxation and decreased amplitude of contraction. The auriculo-ventricular dissociation was still evident at 2 hours, the end of the experiment. In one experiment out of 10, the above concentration caused a systolic effect uncomplicated by conduction impairment and brought the ventricle to a permanent state of two-thirds systole.

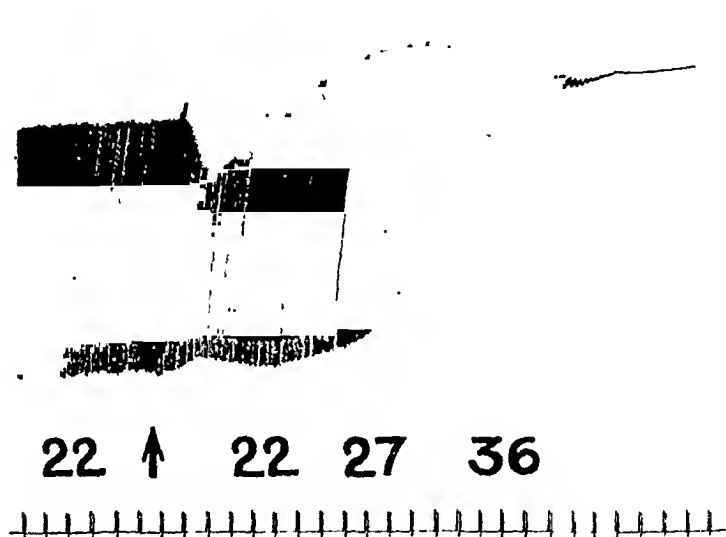


FIG. 4. THE ACTION OF IODOACETAMIDE ON THE ISOLATED FROG HEART  
At arrow, iodoacetamide  $1 \times 10^{-3}$ . The numbers indicate heart rate per minute. Time in 30-second intervals.

In order to obtain ventricular contracture para-chloromercuric benzoate had to be used in concentrations of  $2 \times 10^{-1}$  to  $5 \times 10^{-1}$ . As shown in fig. 6, when these high concentrations were used, a purely depressant effect was immediately produced. This lasted for approximately one minute and was followed by systolic effect. However, before final contracture was achieved, the heart stopped beating. The ventricular contraction was then resumed by means of electrical stimulation. In the experiment as shown in fig. 6, after the heart had stopped beating for approximately 50 seconds, electrical stimulation was applied at a rate of 42 shocks per minute. The decrease in excitability produced by the mercurial compound was so pronounced that the intensity of the stimulus had to be increased (fig. 6, E<sub>1</sub>) in order to drive the ventricle to systolic standstill. This effect could not be reversed by repeated washings with Clark's solution.

Eleven experiments were made with para-chloromercuric benzoate in a concentration of  $2 \times 10^{-4}$ . In only 2 cases complete systolic effect was achieved without electrical stimulation. Of 12 experiments made with a concentration of  $5 \times 10^{-4}$  electrical stimulation had to be applied in all cases. In one experiment with each of the above concentrations the heart stopped beating immediately

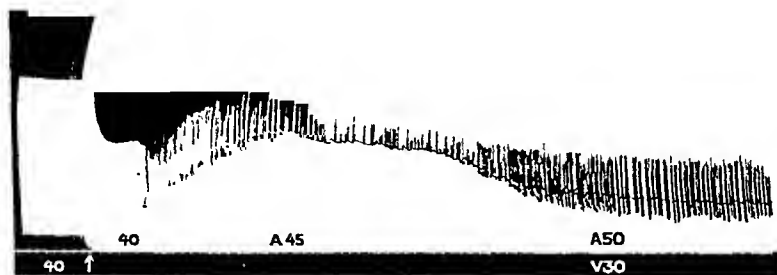


FIG. 5. THE ACTION OF SODIUM PARA-CHLOROMERCURIC BENZOATE ON THE ISOLATED FROG HEART

At arrow, sodium parachloromercuric benzoate  $2 \times 10^{-5}$ . The numbers indicate heart rate per minute. 'A', auricle. 'V', ventricle. Time in 30-second intervals.

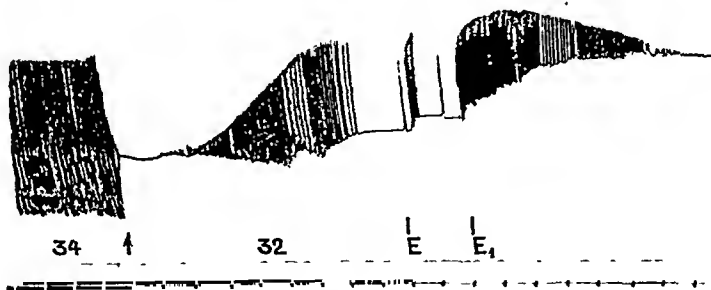


FIG. 6. THE ACTION OF SODIUM PARA-CHLOROMERCURIC BENZOATE ON THE ISOLATED FROG HEART

At arrow, sodium parachloromercuric benzoate  $5 \times 10^{-4}$ . The numbers indicate heart rate per minute. E, electrical stimulation. Time in 30-second intervals.

after applying the compound. Electrical stimulation failed to produce any effect in these 2 cases.

(b) *Three-amino, 4-hydroxy-benzene-arsenous hydrochloride*. Figure 7 illustrates the results obtained with this organic arsenical. A concentration of  $1 \times 10^{-4}$  ( $4.2 \times 10^{-3}$  M) caused an increase in the amplitude of contraction lasting for approximately 15 minutes (fig. 7, A). This was followed by a progressive decrease in the force of contraction which ended in diastolic arrest of the ventricle in approximately 50 minutes. A concentration of  $1 \times 10^{-3}$  (fig.

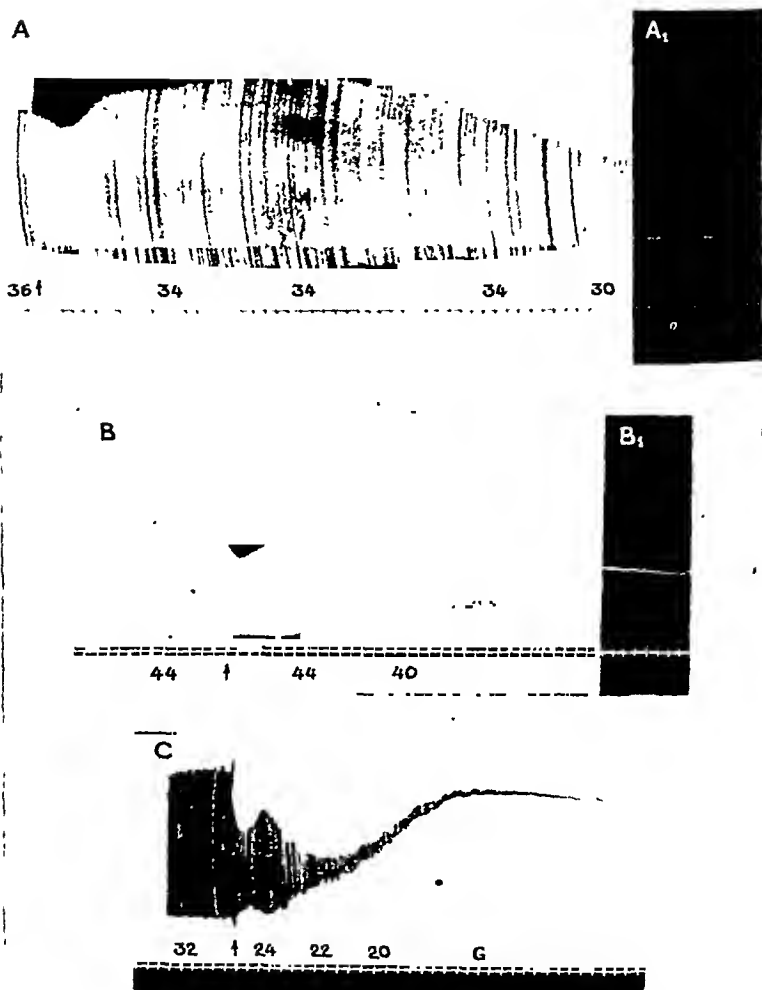


FIG. 7. THE ACTION OF 3-AMINO-4-HYDROXY-BENZENE-ARSENIOUS HYDROCHLORIDE ON THE FROG HEART

Tracing A. at arrow, 3-amino-4-hydroxy-benzene-arsenous hydrochloride  $2 \times 10^{-4}$ . A<sub>1</sub>, 35 minutes after A. Tracing B, at arrow, 3-amino-4-hydroxybenzenearsenous hydrochloride  $1 \times 10^{-3}$ . B<sub>1</sub>, 14 minutes after B. Tracing C. at arrow, 3-amino-4-hydroxybenzenearsenous hydrochloride  $2 \times 10^{-3}$ . G, glutathione. The numbers indicate heart rate per minute. Time in 30-second intervals

7, B) caused a marked depressant effect followed by a slowly developing systolic effect with diminished contraction, terminating in ventricular arrest in a state of approximately two-thirds systole within a period of about 30 minutes. The

degree of final systole obtained with this concentration was not uniform. In some experiments complete systole was obtained, while in others the ventricle stopped in half systole. In order to provoke ventricular contracture a concentration of  $2 \times 10^{-3}$  had to be used. As can be seen in fig. 7, C, this concentration caused an immediate depressant effect followed by a rapidly developing systolic effect which terminated in complete systole within 15 minutes after application.

This arsenical has a tendency to decrease the heart rate. The decrease in excitability caused by the compound was not as pronounced as that produced by para-chloromercuric benzoate, since complete systolic effect of the arsenical when used in a concentration of  $2 \times 10^{-3}$  was obtained without electrical stimulation.

IV. *The effect of glutathione upon the action of sulfhydryl reagents.* (a) *Protective action.* Glutathione protected the heart against the effect of all five reagents studied. Complete protection against the effects of the oxidizing agents was afforded when they were dissolved in a solution of a glutathione which on a molar basis was twice that of the —SH inhibitor. The effect of iodoacetamide was prevented when this reagent was dissolved in a solution of glutathione 5 times as strong as that of the inhibitor. Smaller concentrations of glutathione were not studied. For the protection against the effect of mercaptide-forming compounds, concentrations of glutathione about 10 times that of the —SH inhibitor were used. Not only the systolic effect, but also the diastolic arrest caused by the mercaptide-forming compounds, was prevented by the previous addition of glutathione.

(b) *Irreversibility.* The same concentrations of glutathione used to afford protection against the effect of the —SH inhibitors were employed in an attempt to reverse their systolic effect. In no case could the systolic effect be reversed. Repeated washings with glutathione forcing the solution into the ventricular cavity failed to produce any noticeable effect. In the case of iodosobenzoate and iodoacetamide, glutathione caused a delay in the time necessary to produce systolic standstill, but no reversibility could be obtained. The optimal concentrations for these effects were  $2 \times 10^{-4}$  for iodosobenzoate and  $2 \times 10^{-4}$  to  $1 \times 10^{-4}$  for iodoacetamide. The replacement with glutathione was made at about the middle of the period required for the total effect of the above concentrations of the reagents (see fig. 3). In the case of the arsenical compound, glutathione caused a complete arrest of its systolic effect. This was obtained when the glutathione was replaced at approximately the two-thirds stage of the systolic action.

V. *The effect of BAL.* The slight solubility of BAL in Clark's solution and its toxic effect upon the frog's heart prevented us from studying the action of this reactivator upon the effect of the —SH reagents. A concentration of  $1 \times 10^{-2}$  of BAL caused a depressant effect upon the frog's heart which ended in diastolic arrest in approximately two minutes. The effect of BAL in counteracting the toxic effects of para-chloromercuric benzoate and salyrgan upon the mammalian heart has been studied recently by Long and Farah (6).

DISCUSSION. The three categories of compounds used in this investigation do

not have any chemical relationship among themselves. The only characteristic they have in common is their ability to react with the —SH groups of the sulfhydryl enzymes. This and the fact that the effect of all reagents studied by us could be protected by glutathione suggests that their property to cause diminished relaxation and systolic arrest of the frog heart is due to their property of reacting with the sulfhydryl groups of the sulfhydryl enzymes. No chemical verification of this action has been attempted in this study. The mechanism of the combination between the —SH inhibitors and the enzyme has been discussed by Barron and Singer (1).

Of the two oxidizing agents studied, ortho-iodosobenzoate behaved as a more powerful inhibitor than porphyrindin. This agrees with the results obtained by Barron and Singer (1) in their comparative study of the inhibiting effect of sulfhydryl reagents on succinoxidase.

Iodoacetamide in a concentration of  $1 \times 10^{-4}$  causes systolic standstill in approximately the same time as that of iodosobenzoate in the same concentration. However, the range of concentration of iodoacetamide to produce the same effect is greater than that of iodosobenzoate or porphyrindin. Another difference between iodoacetamide and the oxidizing agents is that the former in small concentrations causes systolic standstill only after a period of arrest of the ventricle in half or one-third systole while the latter in concentration lower than those necessary to produce complete systolic effect cause only a temporary increase in the amplitude of contraction. These differences suggest a more firm combination between the inhibitor and the enzyme in favor of iodoacetamide. This suggestion is substantiated by the fact that in isolated enzyme systems the combination between iodoacetamide and the enzyme is an irreversible one, while oxidation of the —SH groups by the oxidizing agents can be reversed with glutathione.

We have no explanation for our failure to confirm in the isolated frog heart the result of Barron and Singer, according to whom the enzyme is susceptible to reactivation with glutathione after oxidation of the —SH groups with the oxidizing agents or after combination with the mercaptide-forming compounds. The results of Barron and Singer have been confirmed by Long and Farah (6) using mercurial compounds in the heart-lung preparation of the dog.

A detailed study of the effect of both mercaptide-forming compounds has failed to substantiate our preliminary finding (3) that the systolic effect of the arsenical compound is facilitated by placing a copper wire in the fluid of the Straub cannula. The production of systolic standstill with the mercaptide-forming compounds is a matter of using them in the proper concentration (see results).

Barron and Singer divide the protein —SH groups into two types, "freely reactive —SH groups which are readily oxidized and give the nitroprusside test when the protein is in the native state and sluggish —SH groups which are not oxidized and do not give the nitroprusside test except after the denaturation of the protein". The oxidizing agents belong to the first group and the mercaptide-forming reagents, to the latter. Our results show that although both types

of reagents cause systolic standstill of the frog heart, the oxidizing agents and iodoacetamide do it in a way similar in many respects to that of certain peroxides (5) and the cardiac glycosides. However, the effect of the mercaptide-forming compounds is complicated by their depressant action upon the excitability and the contractility of the ventricular muscle. The differences in the results obtained with the two types of reagents are well illustrated in figs. 1, 2, 4, 6, and 7. Whether or not these differences are due to the selective combination of any of the two types of reagents with any of the two types of  $-SH$  groups could only be ascertained by titration of the  $-SH$  groups in the heart. The fact that glutathione protects against both the systolic and the diastolic effects of the mercaptide-forming compounds suggests that the two effects are caused by combination of the reagents with the  $-SH$  groups.

#### SUMMARY

The effect of three groups of specific reagents which combine with the  $-SH$  groups of the sulfhydryl enzymes has been studied on the frog heart. These groups include: (1) two oxidizing agents, porphyrindin and sodium ortho-iodosobenzoate; (2) the alkylating reagent, iodoacetamide; and (3) two mercaptide-forming compounds, sodium para-chloromercuric benzoate and 3-amino, 4-hydroxybenzene arsenous hydrochloride.

All the compounds cause a diminished relaxation of the ventricle which terminates in systolic standstill. In regard to their action upon the frog heart they can be divided into two groups: (1) the oxidizing agents and iodoacetamide, in which the standstill is preceded by an increased amplitude of contraction and a systolic effect resembling the effect of the cardiac glycosides and (2) the mercaptide-forming compounds in which systolic standstill is preceded by a depressant effect caused by a diminished excitability and contractility of the ventricular muscle. In concentrations smaller than those necessary to cause systolic standstill the mercaptide-forming compounds produce a predominantly diastolic effect.

Glutathione protects against the effects of all five reagents, but does not reverse their action.

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# METHIONINE THERAPY IN EXPERIMENTAL LIVER INJURY PRODUCED BY CARBON TETRACHLORIDE\*

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Many experiments have demonstrated that diets high in protein and carbohydrate give maximal protection against various forms of hepatic damage, whereas a high fat diet will increase the degree of liver damage (1, 2, 3, 4, 5, 6). It is also known that a low protein or a protein-free diet will increase the amount of fat in the liver and produce changes in hepatic function that can be prevented by lipotropic substances (7, 8). In 1942 Miller and Whipple reported that the toxicity of chloroform anesthesia for *Protein-depleted* dogs was decreased by the administration of methionine (9). It was of particular interest that the methionine was still effective when given up to four hours after the chloroform anesthesia. Beattie et al reported that therapy with methionine brought about recovery in a patient that had ingested 30 to 40 cc. of carbon tetrachloride (10). However, there are cases in the literature that have survived larger doses of  $\text{CCl}_4$  without methionine therapy (11). More recently methionine has also been reported to be of value in the treatment of hepatitis in industrial workers resulting from inhaling carbon tetrachloride fumes (12), but little critical evidence is given to support this conclusion.

The effect of methionine therapy in the experimental animal and man probably will depend largely on the previous protein intake. The studies of Miller and Whipple with chloroform and methionine were performed with a *protein-free* diet (9). Earlier studies by Bollman did not show any influence of a high protein intake on the survival of rats receiving carbon tetrachloride by inhalation (2). Shaffer and Critchfield studied the sulfur and nitrogen content of the liver of dogs receiving a normal protein intake and  $\text{CCl}_4$ , but did not obtain any indication that sulfur metabolism was increased. They assumed, therefore, that methionine was not required in larger amounts (13). Recent studies by Drill and Loomis (14) and Shaffer et al (15) have not shown any protective effect of methionine against liver damage in dogs produced by  $\text{CCl}_4$  when a *normal* protein diet was fed.

In view of the above reports a more exhaustive study of the therapeutic effect of methionine was made at different levels of protein intake, ranging from sub-normal to above normal, together with various dose levels of  $\text{CCl}_4$  under both acute and chronic conditions. Dogs were utilized, rather than rats, so that serial changes in liver function could be followed throughout the time  $\text{CCl}_4$  was administered. Terminal studies of hepatic pathology were also made.

\* This study was aided by a grant from Eli Lilly and Company and the Fluid Research Fund of Yale University School of Medicine.

**METHODS.** Thirty-four adult mongrel dogs were maintained on the laboratory stock diet and then transferred to a synthetic diet containing 41%, 20%, or 8% casein.<sup>1</sup> All animals were allowed food for a three-hour period each day and the daily food intake was measured and calculated as calories consumed per square meter of surface area per hour. The carbon tetrachloride was administered with an equal volume of corn oil by stomach tube before the dogs were fed. Changes in hepatic function were studied by means of the bromsulphalein and serum phosphatase, which have been shown to be sensitive methods of detecting hepatic dysfunction produced by  $\text{CCl}_4$  (16). Serum phosphatase was determined by the method of Bodansky (17) and bromsulphalein retention by the method of Rosenthal and White (18). After a dose of 5 mgm. of dye per kilo of body weight had been injected, a single blood sample was taken one-half hour later, and the concentration of dye present was divided by two and one-half. Six per cent retention of dye and 5.00 units of phosphatase were taken as the upper limits of normal. The methionine was administered either as an oral supplement or intravenously in doses of 1 gram in 20 to 25 cc. of distilled water. At the conclusion of each experiment liver slices were fixed in Bouin's solution and stained with hematoxylin-eosin, and also in 10% formalin for fat staining with Sudan III.

**RESULTS.** *Experiment 1. High protein intake (41% casein) and 0.5 cc. of  $\text{CCl}_4$  per kilogram.* Dogs 1 to 6 received 0.5 cc. of  $\text{CCl}_4$  per kilo of body weight on days one and five of the study and liver function tests performed on the eighth day. The methionine was administered intravenously in doses of 1.0 gram on the day the  $\text{CCl}_4$  was given and on the following day; namely, on days numbered one, two, five and six of the study. The methionine was injected at this interval as it has been reported that histologically the liver damage with this dose of  $\text{CCl}_4$  is at a maximum in forty-eight hours (19). With the limited amount of methionine available at this time only two dogs were treated. Liver function tests were performed two days after the last dose of  $\text{CCl}_4$ . The data show a wide spread in the response of the control dogs of  $\text{CCl}_4$  (figs. 1 and 4). The two methionine-treated dogs are at the upper limit of the control group, and it was judged that no protective effect of the methionine on the liver was obtained, as determined by the bromsulphalein and serum phosphatase determinations.

*Experiment 2. Normal protein intake (20% casein) and 0.25 cc.  $\text{CCl}_4$  per kilogram.* It was possible that methionine might not exert any protective effect when supplementing a 41% casein diet but might do so when a 20% casein diet was used, which still supplied a normal amount of protein. In this study, therefore, the dogs received the 20% casein diet. The dose of  $\text{CCl}_4$  also was reduced from that in the previous experiment. Dogs 7 and 12 were given 0.25 cc. of  $\text{CCl}_4$  per kilo of body weight on days one, three and five, and liver function tests were performed two days after each preceding dose of  $\text{CCl}_4$  (fig. 2). The

<sup>1</sup> The 20% casein diet consisted of: casein, 20; sucrose, 54.6; lard, 21.5; bone ash, 2.6; salt mixture (Karr) 1.3%. In the 41% and 8% casein diets the per cent of sucrose was correspondingly adjusted. Ten drops of *Oleum percomorphum* were added to each kilo of diet. Four grams of yeast concentrate was administered each day which supplied the following: thiamine, 3.6 mgm.; riboflavin, 640 gamma; pantothenic acid, 1.8 mgm.; nicotinic acid, 8.0 mgm.; pyridoxine, 600 gamma.

The authors wish to thank Mr. R. F. Light of The Fleischmann Laboratories for the yeast extract and Dr. C. E. Bills of Mead Johnson and Company for the *Oleum percomorphum*.

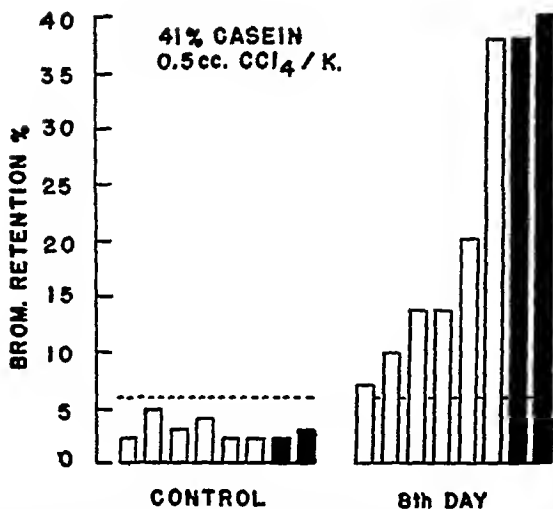


FIG. 1. EFFECT OF METHIONINE ON LIVER FUNCTION OF DOGS RECEIVING  $\text{CCl}_4$ . Open bars are untreated control animals and solid bars indicate methionine treatment. Horizontal broken line shows upper limit of normal of bromsulphalein retention.

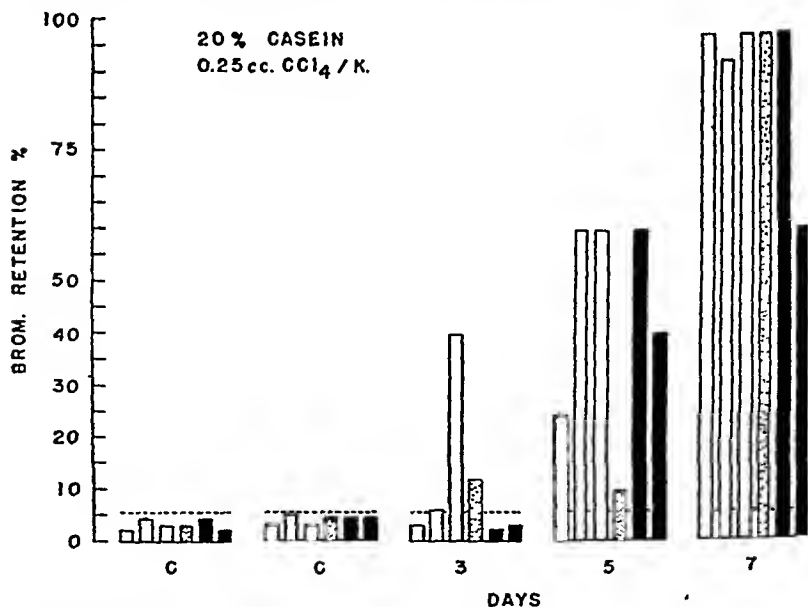


FIG. 2. EFFECT OF METHIONINE ON LIVER FUNCTION OF DOGS RECEIVING  $\text{CCl}_4$ . Open bars indicate untreated control animals; solid bars indicate methionine treatment; stippled bar received only 1 gram of methionine per day. Animals 7 to 12 arranged in numerical order in each set of data.

methionine was administered intravenously. Of the treated animals, dogs No. 11 and No. 12 received 1.0 gram of methionine morning and afternoon each day, starting with the first administration of  $\text{CCl}_4$ . Animal No. 10 received only 1.0 gram of methionine each day. During the period of  $\text{CCl}_4$  administration the food intake of the animals decreased and ranged from 50% to 80% of normal.

The animals were autopsied on the seventh day. Histologically the typical fatty changes of  $\text{CCl}_4$  were observed in all dogs and necrosis in two dogs in each group (table 1). The fat was present both centrally and peripherally, with

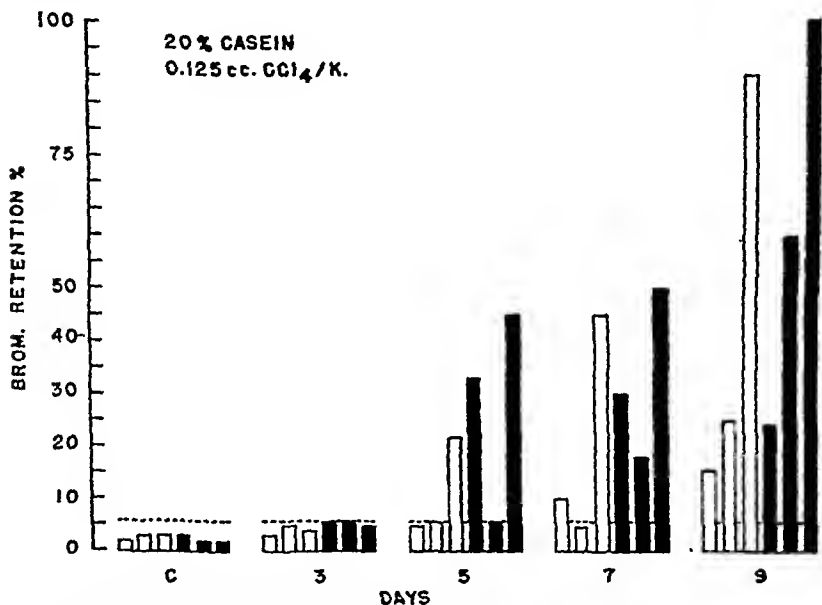


FIG. 3. EFFECT OF METHIONINE ON LIVER FUNCTION OF DOGS RECEIVING  $\text{CCl}_4$ . Open bars indicate untreated control animals; solid bars, methionine treatment. Animals 13 to 18 are arranged in numerical order in each set of data.

accentuation in the central area in animals 7, 8, 9 and 11. In dogs 10 and 12 the fat was only central in location. The changes in liver function are shown in figures 2 and 4.

It was concluded from the functional and histological changes in the liver that supplements of methionine did not provide hepatic protection against  $\text{CCl}_4$  above that already supplied by the 20% casein diet.

*Experiment 3. Normal protein intake (20% casein) and 0.125 cc. of  $\text{CCl}_4$  per kilogram.* It was possible that the dose of  $\text{CCl}_4$  in the previous experiment was too high and might overshadow any effect of the methionine. The dose of  $\text{CCl}_4$  was reduced, therefore, to 0.125 cc. per kilo and administered to dogs 13 to 18 on days one, three, five and seven. Hepatic function tests were performed on days three, five, seven and nine. Animals 16, 17 and 18 received

two grams of methionine per day as in experiment 2. Again, even during the short period that it was administered, the  $\text{CCl}_4$  produced a decrease in food intake and a slight loss of weight in all animals.

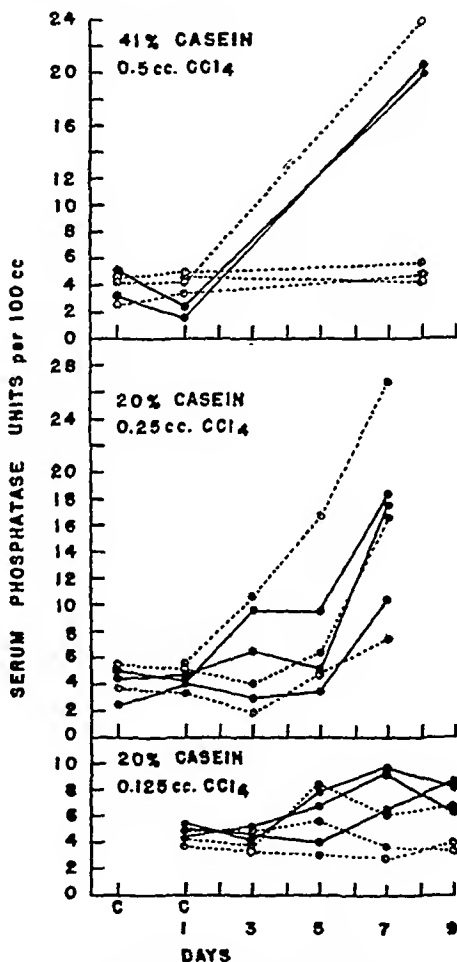


FIG. 4. EFFECT OF METHIONINE ON SERUM PHOSPHATASE OF DOGS RECEIVING  $\text{CCl}_4$  (EXP. 1, 2 AND 3)  
Solid circles indicate methionine treatment; open circles, no treatment

With the lower dose of  $\text{CCl}_4$  there was a somewhat greater spread of data than in the previous experiment, but the results with the methionine are within the range of the untreated animals (figs. 3 and 4). The animals were autopsied on the ninth day for histological studies (table 1). Neither the functional nor

histological studies indicated any protective effect of methionine above that supplied by the 20% casein diet.

*Experiment 4. Normal protein intake (20% casein) during the production of chronic liver damage.* In contrast to the previous experiments, which were acute in nature, a more chronic study was undertaken. Eight animals (No. 19-26) received the 20% casein diet. Carbon tetrachloride was administered in doses of 0.125 cc. per kilo of body weight as in experiment 3, but only twice

TABLE 1  
*Effect of methionine on liver damage produced by carbon tetrachloride*

UNTREATED				METHIONINE TREATED			
Dog number	Fat	Necrosis	Mitosis	Dog number	Fat	Necrosis	Mitosis
Exp. 2, 20% casein, 0.25 cc. CCl <sub>4</sub> /K, acute							
7	+++	+	0	10	+++	+++	+
8	+++	+	0	11	++	0	0
9	++	0	0	12	+++	++	++
Exp. 3, 20% casein, 0.125 cc. CCl <sub>4</sub> /K, acute							
13	+++	0	0	16	+	0	0
14	+	0	0	17	+	+++	0
15	+++	+	0	18	+	+	+++
Exp. 4, 20% casein, 0.125 cc. CCl <sub>4</sub> /K, chronic							
19	0	0	0	23	+	0	0
20	+	0	0	24	++	+	0
21	++	+++	0	25	0	0	0
22	++	0	+	26	++	++++	0
Exp. 5, 8% casein, 0.125 cc. CCl <sub>4</sub> /K, acute							
27	++	0	0	30	++	0	0
28	++	0	0	31	+++	0	0
29	+	0	0	32	++	0	0
Exp. 6, 8% casein, 0.125 cc. CCl <sub>4</sub> /K, acute							
33	+++	0	0	34	++++	0	0

a week instead of every two days. Four of the dogs received 2.0 grams of methionine mixed with their food each day.

By administering this smaller dose of CCl<sub>4</sub> only twice a week the production of liver damage was more gradual (fig. 5). Although there was variation among the untreated animals, it will be seen that the methionine-treated animals always varied within the limits of the untreated dogs. Of the two animals that died during the study both were methionine-treated animals. Dog 26 died on the 21st day and dog 25 on the 25th day of the experiment. The death of these

two grams of methionine per day as in experiment 2. Again, even during the short period that it was administered, the  $\text{CCl}_4$  produced a decrease in food intake and a slight loss of weight in all animals.

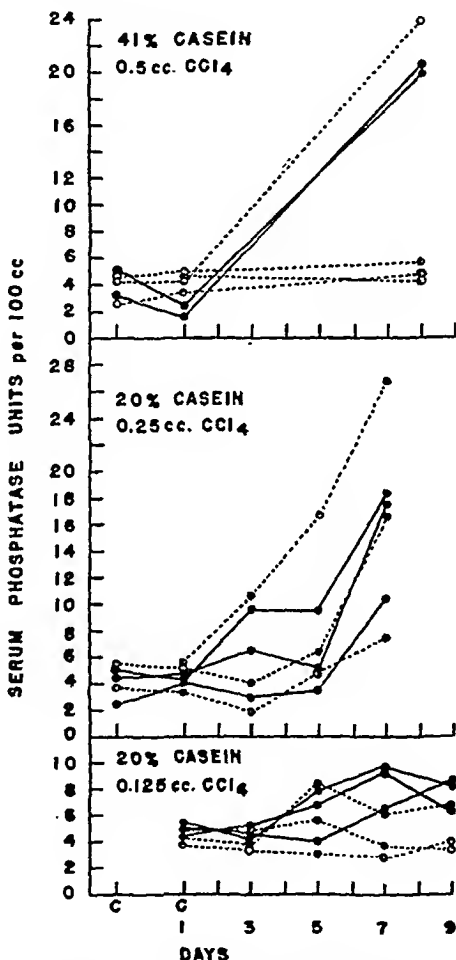


FIG. 4. EFFECT OF METHIONINE ON SERUM PHOSPHATASE OF DOGS RECEIVING  $\text{CCl}_4$  (EXP. 1, 2 AND 3)  
Solid circles indicate methionine treatment; open circles, no treatment

With the lower dose of  $\text{CCl}_4$  there was a somewhat greater spread of data than in the previous experiment, but the results with the methionine are within the range of the untreated animals (figs. 3 and 4). The animals were autopsied on the ninth day for histological studies (table 1). Neither the functional nor

histological studies indicated any protective effect of methionine above that supplied by the 20% casein diet.

*Experiment 4. Normal protein intake (20% casein) during the production of chronic liver damage.* In contrast to the previous experiments, which were acute in nature, a more chronic study was undertaken. Eight animals (No. 19-26) received the 20% casein diet. Carbon tetrachloride was administered in doses of 0.125 cc. per kilo of body weight as in experiment 3, but only twice

TABLE 1

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Exp. 2, 20% casein, 0.25 cc. CCl <sub>4</sub> /K, acute							
7	+++	+	0	10	+++	+++	+
8	+++	+	0	11	++	0	0
9	++	0	0	12	+++	++	++
Exp. 3, 20% casein, 0.125 cc. CCl <sub>4</sub> /K, acute							
13	+++	0	0	16	+	0	0
14	+	0	0	17	+	+++	0
15	+++	+	0	18	+	+	+++
Exp. 4, 20% casein, 0.125 cc. CCl <sub>4</sub> /K, chronic							
19	0	0	0	23	+	0	0
20	+	0	0	24	++	+	0
21	++	+++	0	25	0	0	0
22	++	0	+	26	++	++++	0
Exp. 5, 8% casein, 0.125 cc. CCl <sub>4</sub> /K, acute							
27	++	0	0	30	++	0	0
28	++	0	0	31	+++	0	0
29	+	0	0	32	++	0	0
Exp. 6, 8% casein, 0.125 cc. CCl <sub>4</sub> /K, acute							
33	+++	0	0	34	++++	0	0

a week instead of every two days. Four of the dogs received 2.0 grams of methionine mixed with their food each day.

By administering this smaller dose of CCl<sub>4</sub> only twice a week the production of liver damage was more gradual (fig. 5). Although there was variation among the untreated animals, it will be seen that the methionine-treated animals always varied within the limits of the untreated dogs. Of the two animals that died during the study both were methionine-treated animals. Dog 26 died on the 21st day and dog 25 on the 25th day of the experiment. The death of these



animals was not due to pneumonia or other obvious cause. It was noted that on the 35th and 38th days the bromsulphalein retention of the methionine-treated animals showed a sharp drop towards normal. At first it was thought that some effect of methionine was being obtained, but at this time and on subsequent tests the untreated animals also showed a decreased dye retention. There was again some increase in dye retention after the 45th and 49th days which was maintained until the end of the experiment on the 66th day.

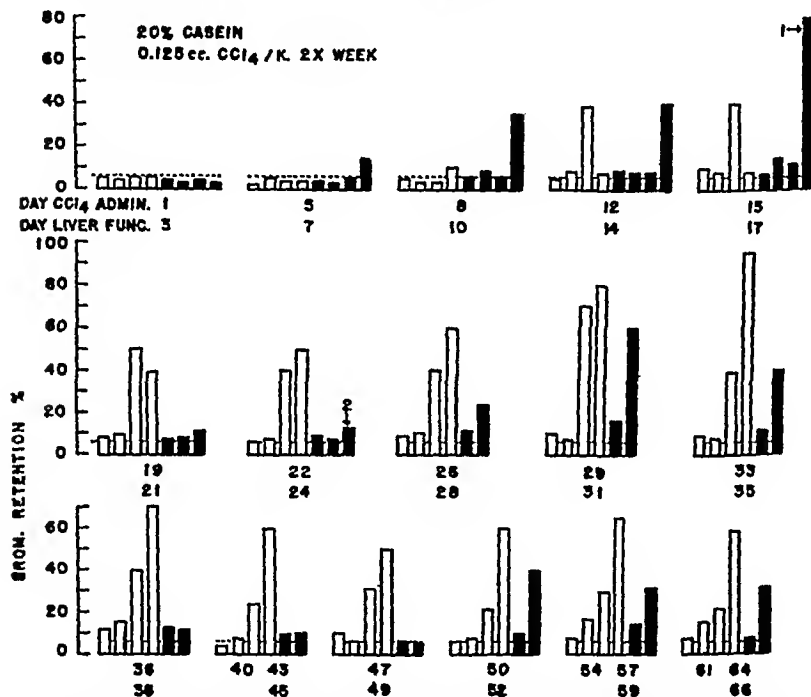


FIG. 5. EFFECT OF METHIONINE ON LIVER FUNCTION OF DOGS RECEIVING CCl<sub>4</sub>

Open bars indicate untreated control animals; solid bars, methionine treatment. Dotted horizontal line indicates upper limit of normal. Animals 19 to 26 are arranged in numerical order in each of data. Arrow 1, dog No. 26 died before next test. Arrow 2, dog 25 died before next test.

The measurements of calorie intake (per square meter of surface area per hour) during this study are of interest (table 2). Dogs 21, 22 and 24, which showed the greatest voluntary decrease in caloric intake, also were the ones which had the largest retention of bromsulphalein at the termination of the experiment. This relationship to dye retention is not due to the decreased food intake *per se*, because we have studied greater degrees of inanition on the same diet for much longer periods of time without observing any effect on dye retention. It cannot be stated from these observations whether the greater hepatic dysfunction

in these animals produced the decrease in food intake, or the voluntary decrease in calorie consumption plus  $\text{CCl}_4$  was a causative factor in producing a greater dye retention.

The histological changes found in the liver of these dogs are listed in table 1. Dog 26, the first animal to die, showed extensive necrosis of the liver; whereas dog 25, which died on the 25 day, showed neither necrosis nor fatty change in the liver. In the two methionine-treated animals that survived until the end of the study, the changes found in the liver were in the same range as those observed in the control animals. Neither the bromsulphthalein retention, serum

TABLE 2

*Weekly food intake as calories/sq. m /hr. of dogs receiving 0.125 cc.  $\text{CCl}_4/\text{K}$  twice a week.*

DOG NO	CONTROL PERIOD*	CALORIES/SQ M /HR CONSUMED, AVERAGE PER WEEK										WEIGHT CHANGE	FINAL DYE RETENTION
		Weeks receiving CCl <sub>4</sub>											
		1	2	3	4	5	6	7	8	9	10		
Untreated dogs													
19		83.0	58.0	56.0	94.5	101.5	93.5	83.0	68.5	64.5	48.5	kgm	%
20	74.1	61.7	66.3	77.3	86.7	88.4	125.0	76.2	79.5	59.1	62.4	+0.9	7
21	72.1	64.8	59.5	69.1	71.8	64.1	91.2	66.8	66.8	29.8	29.2	0.0	15
22	86.5	55.7	54.7	53.6	59.4	69.3	35.4	42.2	44.7	49.7	43.7	-1.6	21
												-1.0	60
Methionine treated													
23	63.1	47.8	36.8	46.0	22.6	73.8	81.7	53.5	57.1	71.2	48.0	-0.2	7
24	72.3	68.1	67.1	48.6	30.6	32.5	84.7	48.5	52.6	45.5	22.5	-2.1	32
25	57.9	37.0	21.1	20.7	21.0							-2.3	12
26	88.1	76.6	23.1	14.7								-1.1	80
Control dogs without CCl <sub>4</sub>													
	68.9	58.2	61.5	71.3	69.9	78.2	73.6	49.8	52.3	72.2	70.3	+0.2	4
	59.8	61.4	56.3	49.1	65.1	69.5	69.1	57.1	87.4	58.0	65.4	+0.5	5

\*Average of four weeks

phosphatase, or the histological studies gave any evidence of a protective effect of the methionine supplements on the liver damage produced by  $\text{CCl}_4$  in this chronic study.

*Experiment 5. Low protein intake (8% casein) and 0.125 cc. of  $\text{CCl}_4$  per kilogram.* The previous studies, both acute and chronic, were performed with a normal protein diet and without any demonstrable effect of methionine on the liver damage produced by  $\text{CCl}_4$ . The experiment was repeated, therefore, using dogs on a low protein diet (8% casein). Following a control period of four weeks on the 20% casein diet six dogs (No. 27-32) were placed on the 8% casein diet for a period of three and one-half weeks. Carbon tetrachloride in doses of 0.125 cc per kilogram, was then administered to all dogs on days

1, 3, 5, 7 and 9, and liver function tests performed two days after each dose of  $\text{CCl}_4$ . During the period of  $\text{CCl}_4$  administration dogs 30, 31 and 32 received 2.0 grams of methionine with their food each day.

Although the methionine-treated dogs were the first to show an increase in bromsulphalein retention, on subsequent tests both groups of dogs showed a similar response (fig. 6). Histologically, sections of the liver taken on the 11th day did not show any necrosis, and there was no significant difference in the degree of fatty change in the liver between the two groups (table 1). Thus, no effect of methionine was observed on either the liver function or histological changes when  $\text{CCl}_4$  was administered to dogs receiving a low protein intake

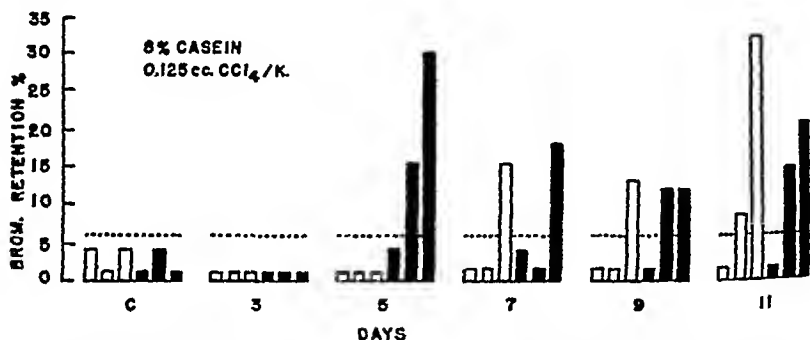


FIG. 6. EFFECT OF METHIONINE ON LIVER FUNCTION OF DOGS RECEIVING  $\text{CCl}_4$ . Open bars, indicate control animals; solid bars, methionine treatment. Dotted horizontal line indicates upper limit of normal. Animals 27 to 32 are arranged in numerical order in each set of data.

*Experiment 6. Low protein intake (8% casein) and 0.125 cc. of  $\text{CCl}_4$  per kilogram.* It seemed desirable to test the effect of methionine on animals fed the 8% casein diet for a longer period of time. Two dogs (No. 33 and 34), therefore, were fed the 8% casein diet for 16 weeks. Carbon tetrachloride was then administered in doses of 0.125 cc. per kilogram on days 1, 3, 5, 7 and 9, and liver function tests were performed two days after each preceding dose of  $\text{CCl}_4$ . Dog No. 33 remained untreated and dog No. 34 received 2.0 grams of methionine intravenously per day during the period of  $\text{CCl}_4$  administration.

When liver function tests were performed on days 3, 5, 7, 9 and 11, the following dye retentions were observed: for dog No. 33 10%, 10%, 34%, 38% and 24% respectively; for dog No. 34 2%, 5%, 14%, 18% and 40% respectively. There was no significant difference between the two dogs. Further, no significant difference can be seen when these data are compared with the dogs (illustrated in Fig. 6) which received the 8% casein diet for only three and one-half weeks. Histologically, dogs 33 and 34 did not show any hepatic necrosis (table 1), although there was some increase in the amount of large fat droplets when compared with the previous experiment.

It was concluded that the feeding of a low casein diet for 16 weeks did not

produce any significant difference in results as compared with dogs receiving a similar diet for three and one-half weeks, and that methionine supplements were without noticeable effect.

**DISCUSSION.** In the above experiments methionine supplements were not observed to have any effect on liver injury produced by carbon tetrachloride as judged by liver function tests and hepatic histology. This was true in animals receiving a normal protein diet or a low protein diet for either three and one-half weeks or sixteen weeks. A similar lack of effect of methionine was obtained in a chronic study with normal protein intake.

These results are in agreement with the recent findings of Drill and Loomis (14) and Shaffer et al (15) using normal protein diets. However, they do not agree with the clinical results reported by Beattie et al (10) and Eddy (12) who reported a beneficial effect of methionine in the treatment of liver injury from carbon tetrachloride. However, in their studies every case was treated with methionine, and additional forms of therapy were used. Therefore, although they report beneficial effects from methionine, adequate evidence is still lacking.

In contrast to the lack of beneficial effects from methionine in  $\text{CCl}_4$  damage in the experiments described in this report, either a high protein intake or methionine supplements seems to have a definite effect on liver damage produced by arsenicals (5, 20), chloroform (4, 6, 9), atabrine (21), ethylene dichloride (22, 23) and propylene dichloride (24). Nevertheless, Bollman has confirmed his original studies with  $\text{CCl}_4$  (2) and again reports, in contrast to the above toxic agents, least liver damage with a diet high in carbohydrate, although regenerative changes were most evident in animals receiving the high protein diet (3). In a discussion of this paper (3) Doctor S. Soskin states that his unpublished results are in agreement with those of Bollman. This problem was also reinvestigated by Gyorgy et al., who studied the inhalation of  $\text{CCl}_4$  in rats and found that a low casein and 10% fat diet increased the mortality, but had little effect on hepatic pathology (25). When the fat was increased to 35% in either the low casein or normal casein diet, hepatic pathology was increased. Although methionine increased the survival rate and suppressed the nephrotic changes completely, "it had only a slight if any beneficial effect on the hepatic injury" (25). Studies in dogs receiving  $\text{CCl}_4$  by stomach tube have also shown a greater degree of hepatic dysfunction when fed a 20% casein diet, as compared with less change when fed an 80% casein diet (26).

In view of the beneficial effects of protein and methionine on other types of liver damage, and a failure to obtain such effects with  $\text{CCl}_4$  damage, it almost seems as if the type of damage produced by  $\text{CCl}_4$  stands in a class by itself. As one of the above-mentioned experimental studies shows, methionine can have a marked effect on survival and on the renal changes produced by  $\text{CCl}_4$ . This emphasized the possibility of toxic effects of  $\text{CCl}_4$  on other organ systems. Indeed, the inhalation of  $\text{CCl}_4$  fumes may produce a clinical picture of mainly pulmonary or renal injury (27) rather than hepatic damage, and death from  $\text{CCl}_4$  fumes may be due to central nervous system damage. At the present time

There is no experimental evidence to show that methionine has a beneficial effect over damage produced by  $\text{CCl}_4$ , even when the protein intake is low. The daily methionine has been reported of benefit in patients with liver damage by  $\text{CCl}_4$ , but the evidence in these papers is not conclusive.

#### SUMMARY

The effect of methionine on the liver injury produced by carbon tetrachloride was studied in dogs, which received both normal and low-casein diets with different doses of  $\text{CCl}_4$ . The amount of fat in the diet was constant in all the studies.

Three *acute* experiments with a normal protein diet (20% and 41% casein) at dose levels of 0.5 cc., 0.25 cc. or 0.125 cc. of  $\text{CCl}_4$  per kilogram were performed. Supplements of methionine were without effect on the dye retention, on phosphatase or pathological changes in the liver.

Methionine was also without effect in preventing or decreasing the degree of liver injury, both functional and histological, in a *chronic* study on dogs receiving a normal protein intake (20% casein) and 0.25 cc. of  $\text{CCl}_4$  per kilogram a week for 66 days.

Supplements of methionine were also without effect on the liver injury produced by 0.125 cc. of  $\text{CCl}_4$  per kilogram in dogs receiving a low protein intake (casein) for three and one-half or 16 weeks, as judged by functional and histological changes in the liver.

At the present time there is no evidence for a beneficial effect on methionine over injury produced by  $\text{CCl}_4$  when either normal or low protein intake are

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# THE METABOLIC FATE OF ACETANILID AND OTHER ANILINE DERIVATIVES

## III. THE ROLE OF p-AMINOPHENOL IN THE PRODUCTION OF METHEMOGLOBINEMIA AFTER ACETANILID

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The generally accepted explanation for the methemoglobinemia following administration of acetanilid and other aniline derivatives is that advanced in 1913 by Heubner (1) and supported in 1942 by Bernheim (2). In this explanation, which is reviewed in the first paper in this series (3), the formation of methemoglobin is attributed to p-aminophenol occurring in the blood as a metabolite. The explanation rests wholly on 3 observed facts: the occurrence of methemoglobin; the presence of p-aminophenol compounds in the blood and urine; and the formation of methemoglobin *in vitro* and *in vivo* from unconjugated p-aminophenol in large amounts. The p-aminophenol recovered from the urine has always been reported as conjugated; its form in the blood has not been determined. It has apparently been assumed without experimental evidence, either that conjugated p-aminophenol forms methemoglobin or that prior to conjugation, p-aminophenol occurs in the blood and in amounts sufficient to produce methemoglobin. In the present paper, these assumptions have been submitted to experimental study.

In his *in vitro* investigation Heubner added p-aminophenol hydrochloride to cows' blood in amounts of and above 25 mg. per cent and observed methemoglobin qualitatively on spectroscopic examination. In the present investigation p-aminophenol hydrochloride was added to freshly drawn oxygenated human blood at room temperature in amounts sufficient to produce 0.1, 0.5, 1.0, 5.0 and 10.0 mg. per cent. Methemoglobin was determined quantitatively (4) at frequent intervals over a period of 4 hours. At concentrations of p-aminophenol of 0.1 and 0.5 mg. per cent no formation of methemoglobin occurred; at a concentration of 1 mg. per cent, 3 per cent methemoglobin developed in one blood sample but none in others; at concentrations of 5.0 and 10.0 mg. per cent, methemoglobin occurred in all samples with average maxima of 13.2 and 21.2 per cent.

It may be concluded that concentrations of p-aminophenol hydrochloride of or in excess of 1.0 mg. per cent are necessary to induce methemoglobin formation *in vitro* at a rate in excess of that of reduction and therefore to permit the accumulation of appreciable amounts of methemoglobin.

In the previous paper in this series (5), it was reported that unconjugated p-aminophenol was not found in the blood of a human subject who had taken 0.975 g. of acetanilid, an amount sufficient to produce in the blood of the subject

approximately 6 per cent of methemoglobin. The method of analysis used there was capable of detecting p-aminophenol at a concentration as low as 0.025 mg. per cent. For the present investigation this feature was again tested using an analytical method modified<sup>1</sup> to increase its sensitivity 5 fold.

A human subject was given 0.975 g. of acetanilid. One hour later the blood showed 4 per cent methemoglobin and at 2 hours 8.2 per cent. At both times no free p-aminophenol was found in the blood indicating that if any was present the concentration was less than 0.005 mg. per cent and therefore definitely below that which produced methemoglobin *in vitro*.

In spite of this finding the possibility remains that the formation of methemoglobin might result from free p-aminophenol which was removed rapidly by conjugation and disappeared before detection by analysis. To investigate this feature p-aminophenol hydrochloride was given to 3 human subjects and the blood analyzed for free and conjugated p-aminophenol and methemoglobin. One subject was given 200 mg. of p-aminophenol hydrochloride; the other two, 500 mg. Blood was drawn for analysis  $\frac{1}{2}$  and 1 hour later. The results are shown in table 1. In the first subject no methemoglobin and no unconjugated p-aminophenol was found. That the p-aminophenol was rapidly absorbed and also rapidly conjugated was shown by the occurrence in the blood at  $\frac{1}{2}$  hour of 0.41 mg. per cent of total conjugated p-aminophenol. In the two subjects given 500 mg. of p-aminophenol no methemoglobin was found but unconjugated p-aminophenol was present in the blood at  $\frac{1}{2}$  and 1 hour.

The results obtained show that free p-aminophenol in amounts detectable by analysis may occur in the blood without the formation of methemoglobin. It is apparent that the methemoglobin appearing after the administration of 0.975 g. of acetanilid cannot be caused by the transient occurrence of amounts of free p-aminophenol sufficiently large to produce methemoglobin.

As shown in previous work (5), the major metabolites of acetanilid appearing in the blood are N-acetyl p-aminophenol and its hydroxy conjugates with sulfuric and glycuronic acids. The possibility that these compounds are responsible for the methemoglobinemia following administration of acetanilid was investigated. Two normal human subjects in a postabsorptive state were each given orally 1.09 g. of N-acetyl p-aminophenol, equivalent to 0.97 g. of acetanilid. Blood samples were drawn after 1, 2, 4 and 6 hours and analyzed for methemoglobin. In neither subject was any methemoglobin formed. In one subject each blood sample was also analyzed for N-acetyl p-aminophenol and total p-aminophenol. The results are shown in table 2.

Several significant features are apparent from these data. In previous work (3) it has been shown that very little of the N-acetyl p-aminophenol formed in the body from acetanilid is excreted. Most of it is ultimately conjugated on

<sup>1</sup> A 1:2 filtrate was prepared by adding 10 cc. of blood to 10 cc. of 2 N HCl containing 0.75 g. of zinc dust, mixing, and adding 3 g. BaSO<sub>4</sub>. After again thoroughly mixing, 2 drops of capryl alcohol were added and the mixture was centrifuged. Ten cc. of filtrate were drawn through a cotton plug into a pipette and used as described (5) for the estimation of p-aminophenol.



the hydroxy group and eliminated in this form. Six hours after the administration of N-acetyl p-aminophenol in the present experiments, a considerable part was still present, as such, in the body, as indicated by the concentration found in the blood. Considerable time is therefore necessary for the complete conjugation of the hydroxy group of this compound in the body. In contrast to this, 1 hour after administration of 500 mg. of p-aminophenol hydrochloride, as shown in table 1, virtually all of the p-aminophenol found in the blood is already conjugated.

TABLE 1

*Administration of p-aminophenol hydrochloride to human subjects*

SUBJECT	p-AMINOPHENOL HYDROCHLORIDE GIVEN	p-AMINOPHENOL IN BLOOD, MG. %					
		Unconjugated		N-acetyl		Total	
		$\frac{1}{2}$ hr.	1 hr.	$\frac{1}{2}$ hr.	1 hr.	$\frac{1}{2}$ hr.	1 hr.
1	200	0	0	—	—	0.41	—
2	500	0.09	0.012	0.56	0.45	0.84	0.96
3	500	0.12	0.016	0.56	0.26	0.98	0.69

TABLE 2

*Administration of 1.09 g. of N-acetyl p-aminophenol to a human subject*

TIME	p-AMINOPHENOL IN BLOOD, MG. %	
	N-acetyl	Total
hr.		
1	1.19	1.54
2	1.14	1.64
4	0.91	1.23
6	0.53	0.80

The maximum concentrations of N-acetyl p-aminophenol and hydroxy conjugates occurring in the blood after administration of the N-acetyl p-aminophenol are, without formation of methemoglobin, higher than the maximum concentrations of these found after administration of 0.975 g. of acetanilid with formation of methemoglobin. It may be concluded that the methemoglobinemia following acetanilid administration is not due to conjugated p-aminophenol formed in the body.

The question of methemoglobin formation after large doses of N-acetyl p-aminophenol was investigated by oral administration of this compound to albino rats in doses of 1000, 2000 and 4000 mg. per kg. of body weight. Frequent determinations of methemoglobin were made over a period of 8 hours. No methemoglobin was formed in any of the animals. With a dose of 4000 mg. per kg. death resulted with 24 hours. Although the white rat is not a sensitive methemoglobin-forming species, it has been shown (6) that this animal forms approximately 13 per cent methemoglobin after oral administration of 200 mg. per kg. of acetanilid.

It has previously been shown (3) that for several hours following the ingestion of 1 g. of acetanilid in man the urine contains a considerable concentration of conjugated p-aminophenol, the major part of which is hydroxy conjugated. By the addition of 0.2 cc. of urine thus obtained and suitably diluted to each of several 2 cc. portions of freshly drawn human blood, it was possible to obtain concentrations in the blood samples of approximately 5 and 10 mg. per 100 cc. of conjugated p-aminophenol. Frequent determinations in each of these blood samples over a 4-hour period showed no formation of methemoglobin. The concentrations of the conjugated p-aminophenol obtained in the blood samples were many fold higher than that occurring *in vivo* after administration of 0.975 g. of acetanilid. These data offer further evidence that the methemoglobinemia resulting from this amount of acetanilid is not caused by the conjugated p-aminophenol.

#### SUMMARY

- (1) Contrary to the usually accepted theory, the occurrence of methemoglobin after administration of acetanilid is not due to p-aminophenol.
- (2) Concentrations of p-aminophenol at or above 1 mg. per cent are required to produce methemoglobin in human blood *in vitro*.
- (3) No free p-aminophenol was found in the blood after administration of acetanilid although methemoglobin was found.
- (4) Free p-aminophenol was found in the blood after administration of the substance but no methemoglobin was found.
- (5) Conjugated p-aminophenol does not *in vivo* or *in vitro* produce methemoglobin.

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# URINARY AND FECAL EXCRETION OF SULFANILAMIDE DERIVATIVES AFTER ORAL ADMINISTRATION TO RATS<sup>1</sup>

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The therapeutic effectiveness of sulfanilamide derivatives depends, firstly, on the bacteriostatic activity of the compound, and secondly, on the presence of a sufficiently high concentration at the proper site. For treatment of blood-borne diseases, a therapeutic level of the agent must, of course, be maintained in the blood stream; the natural corollary of this is that, to be effective on oral administration, the compound must be largely absorbed from the intestinal tract. Conversely, the therapy of intestinal infections requires that an adequate concentration of the material be present in the intestinal tract as well as in the tissues; thus, the compound should not be too rapidly and completely absorbed following administration by mouth.

A survey of the literature shows that complete excretion studies have been carried out on relatively few compounds, and that in some cases marked discrepancies occur in the values reported. Typical data are presented in table 1.

The purpose of the present investigation was to determine the route of excretion of a number of compounds following oral administration. The results should be useful in considering possible therapeutic applications of any compounds in the series that show promise of effectiveness.

**METHOD.** Adult albino rats were used in the study. All compounds were administered by stomach tube, and those not soluble in water were given in suspension in a 5% solution of gum acacia. The rats received a volume of 2 cc., containing 100-400 mgm. of the material. The animals were placed immediately in metabolism cages with stock ration<sup>2</sup> and water *ad libitum*. The urine and feces were collected and analyzed 24 and 48 hours after administration. Since it was found in preliminary work that insignificant amounts were excreted after 48 hours, collections were discontinued at that time.

The colorimetric method of Bratton and Marshall (27) was used for the analyses. Readings were made in the Fisher Electrophotometer with a No. 525 filter.

Since many of the compounds in the series were substituted in the N4 position, it was necessary to subject them to hydrolysis prior to diazotization and coupling. This was routinely carried out by heating the samples in normal hydrochloric acid on the steam bath for one hour.

Urine samples were diluted to 100 cc. and filtered, and suitable aliquots were taken for hydrolysis. The volume of the hydrolysate required for colorimetry was so small that interference of the colored solution was negligible.

Treatment of the feces depended on the solubility of the compound in question. Those readily soluble in acetone were removed from the feces by thorough Soxhlet extraction

<sup>1</sup> Reported at a meeting of the Federation of American Societies for Experimental Biology, Chicago, May 18-22, 1947.

<sup>2</sup> "Harlan Special Rat Ration" obtained from Harlan Small Animal Industry, Cumberland, Ind.

TABLE 1  
Excretion of sulfanilamide derivatives after oral administration  
A literature survey

COMPOUND	PER CENT OF ORAL DOSE EXCRETED IN		COLLECTION PERIOD	SPECIES	REFERENCE
	Urine	Feces			
			days		
Sulfanilamide	88-97	Negligible	Several	Human	1
	63-78	—	1	Human	2
	41-68	12	1	Human	3
	43	—	1	Mouse	4
	45	—	—	Rat	5
Sulfapyridine	95-99	—	2	Dog	6
	90	—	—	Dog	7
	85	—	2	Human	8
	39-79	—	4	Human	9
	60-65	—	1.5	Human	10
	70	—	1.5	Guinea pig	11
	45-90	2-33	5-12	Human	12
	62	3	4	Mouse	12
	37	—	1	Mouse	4
	65-88	—	5-6	Human	13
Sodium sulfapyridine	19-31	32-54	3	Human	3
Sulfadiazine	79-80	—	2.5	Human	14
	58-81	—	2	Human	15
	20	—	1	Mouse	4
	62-88	—	3	Human	16
	66	—	4	Human	28
	—	56	—	Monkey	28
Sulfamerizine	78	—	4	Human	28
	—	33	—	Monkey	28
Sulfathiazole	46-98	—	8	Human	17
	60-93	—	1	Human	18
	4-11	46-72	—	Human	3
	44	—	1	Mouse	4
	53-99	—	—	Human	19
	18-35	—	5	Human	20
Sodium sulfathiazole	19-25	38-63	2	Human	3
Sulfamethylthiazole	2	38	—	Human	3
Sodium sulfamethylthiazole	8-23	18-78	2	Human	3
Succinylsulfathiazole	2	—	5	Human	20
Sulfaguanidine	32	—	1	Mouse	4
	34	14	2	Human	21
	16-34	—	5	Human	20
Sulfathiadiazole	60-100	4-8	3-7	Human	22
Sulfamethylthiadiazole	96-100	—	—	Human	22
Sulfacarboxythiazole	3-7	83-92	—	Human	23
Sulfamethazine	67-99	—	4	Human	24
Acetylsulfanilamide	30-50	—	1	Rat	25
p-Sulfanilysulfanilamide	25	20	—	Rat	25
Sodium sulfanilamide benzoate	60	—	—	Rat	25
N <sub>1</sub> -Benzoylsulfanilamide	79	—	3	Human	26

TABLE 2  
*Excretion of sulfanilamide derivatives*

COMPOUND	SOLVENT USED IN FECES EXTRACTION	DOSE	PER CENT OF ADMINISTERED DOSE RE- COVERED IN EXCRETA IN 48 HOURS	PER CENT OF RECOVERED AMOUNT FOUND IN	
				Urine	Feces
Sulfanilamide	Acetone	<i>mgr.</i> 105.3	76	96	4
		100.0	40	99	1
		100.0	49	98	2
Sulfaguanidine	Acetone	98.2	55	38	62
		101.7	52	50	50
		100.0	63	32	68
N <sub>1</sub> -Benzoylsulfanilamide	Acetone	100.0	84	88	12
		100.0	73	89	11
		83.0	50	98	2
		83.0	73	95	5
N-(4-Aminobenzenesulfonyl)-2-phenyl- propylamine*	Acetone	100.0	52	10	90
		100.0	84	5	95
		100.0	62	13	87
		100.0	62	15	85
N-(4-Aminobenzenesulfonyl)-N-methyl- 2-phenylpropylamine†	Acetone	406.4	89	5	95
		203.1	85	12	88
		304.2	66	6	94
		100.2	85	2	98
N-(4-Aminobenzenesulfonyl)-ephedrine*	Acetone	100.0	26	23	77
		100.0	52	17	83
		100.0	38	26	74
N-(4-Acetylaminobenzenesulfonyl) N-methyl-2-phenylpropylamine†	Acetone	121.8	50	48	52
		101.8	70	63	37
		101.8	67	46	54
4-Sulfonamidodiphenylthiourea‡	Acetone	149.8	56	4	96
		106.8	48	4	96
		95.6	48	12	88
		155.0	60	3	97
2-(4-Aminobenzenesulfonyl)acetamide§	Acetone	97.8	72	67	33
		94.9	75	93	7
		19.3	58	97	3
Tartarylsulfanilamide	Acetone	100.0	92	5	95
		100.0	102	3	97
Succinylsulfathiazole	Alkali	100.0	80	4	96
		100.0	82	3	97
		100.0	90	3	97
		100.0	89	3	97
		100.0	79	6	94

TABLE 2—*Concluded*

COMPOUND	SOLVENT USED IN FECES EXTRACTION	DOSE	PER CENT OF ADMINISTERED DOSE RE- COVERED IN EXCRETA IN 48 HOURS	PER CENT OF RECOVERED AMOUNT FOUND IN	
				Urine	Feces
		<i>mgm.</i>			
Diacetyltartarylsulfadiazine	Alkali	100.0	88	5	95
		100.0	89	5	95
		100.0	75	9	91
		100.0	96	17	83
Diacetyltartarylsulfathiazole	Alkali	100.0	83	6	94
		100.0	82	7	93
		100.0	78	9	91
		100.0	72	17	83
Sodium propionylsulfanilamide- $\beta$ - sulfonate	Alkali	100.0	74	7	93
		100.0	73	7	93
		100.0	85	5	95
		100.0	77	5	95
Sodium propionylsulfathiazole- $\beta$ - sulfonate	Alkali	100.0	79	5	95
		100.0	70	1	99
		100.0	83	2	98
Sodium 2-sulfobenzoylsulfathiazole	Alkali	100.0	51	6	94
		100.0	67	2	98
		100.0	68	4	96
		100.0	63	3	97

Acknowledgment is to be made to the following for preparing the compounds indicated by signs as follows:

\* Dr. Charles H. Tilford, of The Wm. S. Merrell Co.

† Mr. Lloyd Allen, of The Wm. S. Merrell Co.

‡ Dr. Ralph W. Bost, of the University of North Carolina.

§ Dr. Ralph Connor, formerly of the University of Pennsylvania, and now of the Rohm and Haas Co.

|| Dr. Joseph R. Stevens, of the J. T. Baker Chemical Co.

with this solvent. Several compounds containing carboxylic or sulfonic acid substituents were only very slightly soluble in acetone but were conveniently extracted by stirring with several volumes of normal sodium hydroxide and centrifuging, and repeating the process several times. In either case, virtually water-clear solutions were obtained when aliquots of the extracts were heated with hydrochloric acid and filtered.

With most of the compounds, recovery experiments gave reasonably satisfactory results. The principal exceptions are discussed in the following sections.

RESULTS. Several known compounds were included in the series for comparison with the new derivatives studied. The results, presented in table 2, show that sulfanilamide and  $N_1$ -benzoylsulfanilamide (sulfabenzamide) are excreted principally in the urine, succinylsulfathiazole appears almost entirely in the feces, while sulfaguanidine is rather equally distributed between the two.

Of the new compounds, only one (2-(4-aminobenzenesulfonyl)acetamide)

appeared primarily in the urine; it is to be noted that this is not a true sulfonamide. A second compound (N-4-acetylaminobenzenesulfonyl)-N-methyl-2-phenylpropylamine) was divided fairly equally between urine and feces, while the remaining ten appeared largely in the feces, indicating poor absorption.

TABLE 3  
*Compounds refractory to hydrolysis*

COMPOUND	HYDROLYSIS CONDITIONS	RECOVERY PER CENT
N <sub>1</sub> -(4-n-Dodecoxyphenyl)sulfanilamide*	10 N H <sub>2</sub> SO <sub>4</sub> , over micro burner, 4 hours	0
4-Sulfonamido-4-n-hexoxythiocarbanilide*	conc. HCl, steam bath, 6 hours	38 66
Tri-isobutylenesuccinylsulfathiazole†	N HCl, steam bath, 1 hour	46 55
N <sub>4</sub> -Acid diphenate of sulfathiazole‡	N HCl, steam bath, 1 hour conc. HCl, steam bath, 1 hour conc. HCl, autoclaved 15 lbs., 1 hour 6 N HCl, autoclaved 15 lbs., 6 hours	12 44 75 85
o-Sulfobenzoyl-sulfanilamide‡	N HCl, steam bath, 1 hour	0
Sodium 2-sulfobenzoylsulfanilamide‡	N HCl, steam bath, 1 hour N HCl, steam bath, 3 hours 10% NaOH, steam bath, 2 hours 10 N HCl, steam bath, 2 hours 10 N H <sub>2</sub> SO <sub>4</sub> , autoclaved 15 lbs., 2 hours	0 6 0 65 65
Di-sodium 2-sulfobenzoylsulfathiazole‡	3.6 N HCl, steam bath, 1 hour	7
Sodium 2-sulfobenzoylsulfathiazole‡	4 N HCl, steam bath, 1 hour conc. HCl, steam bath, 20 hours 12 N H <sub>2</sub> SO <sub>4</sub> , refluxed, 77 hours	0 55 82

Acknowledgment is to be made to the following for preparing the compounds indicated by signs as follows:

\* Dr. Ralph W. Bost, of the University of North Carolina.

† Dr. Charles H. Tilford, of The Wm. S. Merrell Co.

‡ Dr. Joseph R. Stevens, of the J. T. Baker Chemical Co.

The series is not adequate for general conclusions as to the relation between structure and absorption. It is interesting, however, that acetylation in the N-4 position greatly increases the rate of absorption of a compound that is otherwise poorly absorbed. Introduction of the tartaryl or diacetyltartaryl group in the N-4 position appears to have the same effect as introduction of the succinyl radical, namely, a marked decrease in absorption from the intestinal tract.

*Isothiocyano compounds.* The series included two compounds in which the p-amino group was replaced by an isothiocyano substituent. The compounds, 4-isothiocyano-benzenesulfanilyl guanidine and 2-(4-isothiocyanobenzene-sulfonamido) pyrimidine, behaved quite similarly and may be considered together.

The compounds were soluble in acetone and, after hydrolysis, gave theoretical values with the usual colorimetric method. However, attempts to recover added amounts from normal feces gave only 30 to 60 per cent of the expected values. As a check on the extraction procedure, it was found that satisfactory results were obtained when the compound was extracted by Soxhlet from an inert diluent (sea sand). It was then observed that addition of the compound to filtered acetone extracts of normal feces, followed by hydrolysis and color development, gave variable but low (30-70%) recoveries; this indicates that the loss cannot be attributed to physical adsorption, in an unextractable form, on the feces particles. Removal of the interfering substance was demonstrated by exhaustive extraction of feces with acetone; addition of the compound to the residual material permitted satisfactory extraction and assay.

Further studies indicated that the interfering substance is readily soluble in acetone, somewhat soluble in ethyl ether, and insoluble in petroleum ether. It is adsorbable by Norite from acetone or ether.

The nature of this phenomenon is still unknown.

*Compounds refractory to hydrolysis.* Eight of the compounds available for study gave little or no color following the usual hydrolysis procedure. More rigorous treatment led to greater recovery in those instances where it was tried. In only one case was a careful study of hydrolysis conditions made; it was found that consistently high results could be obtained by refluxing sodium o-sulfobenzoylsulfathiazole with ten-normal sulfuric acid for 77 hours. Animal experiments were carried out with this compound, and the results are included in table 2.

These difficultly hydrolyzed compounds, along with the conditions employed and recoveries found, are listed in table 3.

#### SUMMARY

An investigation has been made of twenty previously unreported sulfanilamide derivatives.

Following oral administration to rats, ten of the compounds are excreted largely in the feces, one chiefly in the urine, and one is almost equally divided between the two materials.

Two 4-isothiocyanobenzenesulfonamides gave interesting and unexpected results. There appears to be a substance in normal rat feces, and in acetone extracts thereof, which in some way prevents full color development by these compounds after hydrolysis.

The remaining seven compounds are hydrolyzed with difficulty, and a satisfactory analytical method has not yet been worked out.

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# PROTECTIVE ACTION OF p-AMINOBENZOIC ACID AGAINST CERTAIN BISMUTH PREPARATIONS<sup>1</sup>

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In studies reported by Sandground (1, 2), p-aminobenzoic acid (PABA) was found to be highly effective as a protective agent against lethal doses of carbarsone and other phenyl arsonates, namely, tryparsamide, acetarsone, arsanilic acid, and phenyl arsonic acid. In other papers, Sandground and Hamilton (3, 4, 5, 6) reported that PABA also gave a substantial degree of reduction in the mortality rate following injections of lethal doses of neoarsphenamine and the antimony compound, stibosan, without diminution of trypanocidal activity in the rat. PABA was found, however, to be without protective value against inorganic arsenic or certain of the trivalent arsenicals, such as mapharsen and arsphenamine, and against the trivalent antimonial compound tartar emetic.

Subsequently, Sandground reported experiments (2) which demonstrate that several aromatic compounds have the property of reducing the toxicity of pentavalent aromatic arsenicals. The three isomeric forms of aminobenzoic acid, the hydroxy- and nitro-analogues of PABA, and several other phenyl substituted compounds not highly toxic in themselves were found to be substantially effective. A high degree of structural similarity between antidote and the toxic compound does not appear from these reports to be an essential of the mechanism underlying the detoxication phenomenon, and the protective properties were found to be independent of the route by which either the acid or the arsenical was administered. Adequate doses of PABA, administered simultaneously with or at time intervals up to three hours before an arsenical injection, conferred significant protection. In contrast, the injection of the PABA subsequent to the arsenical was associated with a distinct reduction in protective value, and this reduction was in direct proportion to the increase in time interval (7). Multiple injections of PABA given at variously spaced intervals previous to or following the arsenical were found to be no more effective than a single protective dose given concurrently.

Harris (8) presented histologic findings which indicated a lessened severity of renal damage in rats receiving PABA in protective dosage against highly toxic doses of tryparsamide and stibosan.

Sandground found 500 milligrams of PABA per kilogram of weight sufficient to protect nearly all rats against 1000 mg. of carbarsone per Kg. (5). While no unprotected rat was ever found to survive a dose of 1500 mg./Kg. of carbarsone, a single injection of 500 mg./Kg. of PABA was capable of conferring pro-

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tection if administered at a suitable time with reference to the administered arsenical. As small an amount as 15 mg./Kg. was found sufficient to protect 50 per cent of rats receiving 1000 mg./Kg. (the LD<sub>50</sub>) of carbarsonne, and to prolong the survival time of a group of rats receiving 400 mg./Kg. of arsanilic acid.

TABLE 1

*Summary of results with sodium bismuthyl citrate (The Upjohn Co.) alone and with 1.5 cc. of 4 per cent PABA solution intraperitoneally, except when both the bismuth compound and PABA were given intravenously*

ANIMAL GROUP	NUMBER OF RATS USED	ROUTE OF Bi ADMINISTRATION	DOSE OF BISMUTH		PER CENT SURVIVING	
					With PABA	Without PABA
			mg. per Kg.			
(1)	Test rats 5 Controls 5	Intraperitoneal Intraperitoneal	60	60	80	00
(2)	Test rats 4 Controls 4	Intraperitoneal Intraperitoneal	70	70	75	00
(3)	Test rats 4 Controls 4	Intraperitoneal Intraperitoneal	70	70	50	00
(4)	Test rats 3 Controls 3	Intraperitoneal Intraperitoneal	70	70	00	00
(5)	Test rats 5 Controls 5	Intraperitoneal Intraperitoneal	60	60	40	00
(6)	Test rats 3 Controls 3	Intravenous Intravenous	9	9	100	00
(7)	Test rats 5 Controls 5	Intravenous Intravenous	10	10	40	00
(8)	Test rats 5 Controls 5	Intravenous Intravenous	9	9	40	00
(9)	Test rats 5 Controls 5	Intravenous Intravenous	9	9	40	20
(10)	Test rats 3 Controls 3	Intravenous Intravenous	9	9	100	33

In this laboratory a number of experiments have been carried out with PABA, using the rat, with the objective of determining whether or not this acid confers protection against other organo-metallic compounds and to seek an explanation of the way in which protective activity may be conferred. Sandground's findings with respect to the protective action of PABA against the arsenicals were readily confirmed. Efforts also were made to determine whether or not PABA exerts

an accelerating effect on the urinary excretion of arsenic. Doses of carbarsone well below 1000 mg./Kg. (the LD<sub>50</sub>) were given by intraperitoneal injection followed immediately by an intraperitoneal injection of approximately 400 mg./Kg. of PABA. The first 24-hour urine specimen of each rat was analyzed for arsenic content using a method recommended by Young and Muehlberger (9), by which the arsenic as arsenite, after Kjeldahl digestion and neutralization of the excess sulfuric acid with sodium hydroxide and sodium bicarbonate, was titrated with N/20 iodine solution. Some of the unprotected rats receiving the higher doses of arsenic died before the end of the 24-hour period, and, as was to be expected, in many of these cases the urine samples gave a low yield of arsenic.

TABLE 2

*Summary of results of toxicity studies on rats receiving sodium bismuthyl mannonate (Chas. Pfizer and Company) (8 mg. of bismuth per Kg. weight) and an equal dose of sodium bismuthyl mannonate concurrently with 1.5 cc. of 4 per cent PABA solution. Both solutions were given intravenously*

ANIMAL GROUP	NUMBER OF RATS USED	PER CENT SURVIVING	
		With PABA	Without PABA
(1)	Test rats 5 Controls 5	60	20
(2)	Test rats 4 Controls 4	100	50
(3)	Test rats 5 Controls 5	80	20
(4)	Test rats 5 Controls 4	60	50
(5)	Test rats 5 Controls 5	100	40

Rats protected with PABA were usually much less obviously intoxicated by the arsenical than were the unprotected animals, and their arsenic output was generally uniform. In the lower dosage ranges of arsenic, however, where the unprotected rats were not made noticeably ill, the controls were found not to vary on an average from the protected groups in arsenic output. Data accumulated from a large number of 24-hour urine specimens therefore revealed no significant change in output of arsenic by rats receiving PABA, if the dose of arsenic was not large enough to produce a severe reaction.

Since, to our knowledge, no reports have appeared on the protective effects of PABA against the organic bismuth compounds, we have extended our studies into this group of drugs. Young rats ranging in weight from 100 to 150 grams were used. Control series of rats received highly toxic doses of bismuth alone, while the paired series received, in addition to the bismuth, the same protective

dose of PABA as was used against the arsenicals. Our results demonstrate that PABA does confer some protection against sodium bismuthyl citrate, and sodium bismuthyl mannonate, and, to a lesser degree, against sodium bismuthyl tartrate. Whereas various dosages of PABA were used in experiments not reported herein, it was found that doses of 400 to 500 mg./Kg. were somewhat better than smaller doses but not significantly exceeded by those which were larger; hence these doses were adopted for our purposes.

TABLE 3

*Results of intravenous injections of sodium bismuthyl tartrate (G. D. Searle and Company), in rats, with and without simultaneous administration of PABA*

ANIMAL GROUP	NUMBER OF RATS USED	DOSE OF BISMUTH <i>mg. per Kg.</i>		PER CENT SURVIVING	
				With PABA	Without PABA
(1)	Test rats 5 Controls 5	8	8	80	20
(2)	Test rats 4 Controls 4	8	8	100	50
(3)	Test rats 5 Controls 3	10	10	100	00
(4)	Test rats 5 Controls 5	10	10	00	00
(5)	Test rats 5 Controls 5	10	10	00	00
(6)	Test rats 5 Controls 5	8	8	40	20
(7)	Test rats 5 Controls 5	8	8	60	20
(8)	Test rats 5 Controls 5	8	8	40	20

When sodium bismuthyl citrate was given alone by intraperitoneal injection, 40 mg./Kg. of bismuth, calculated as metal, was found nearly always to be fatal. By use of PABA, however, it was found possible to save a high percentage of the rats given 70 mg./Kg. of bismuth as the citrate (see table 1).

Eight milligrams per kilogram of bismuth in the form of sodium bismuthyl mannonate was found to be lethal to a high percentage of rats, and, accordingly, this dose was employed in the few trials which were made with this compound. In table 2 are presented evidences of a significant degree of protection by PABA against bismuth in this form.

Sodium bismuthyl tartrate is slow in action and massive doses are required to

kill the rat when it is given by intramuscular or intraperitoneal injection. Only by intravenous injections were we able to demonstrate what appears to be a rather low order of protection by PABA. By this route it was found that 10 mg./Kg. of bismuth in the form of the tartrate was nearly 100 per cent fatal to the rats. In several series in which less than 10 mg. were used as the trial dose, there was some protection, as indicated by a reduced mortality rate, and less severe reactions in the protected rats (see table 3).

It should be stated that the above tables on mortality do not include other significant features, e.g., the average survival times of the protected groups, which were longer with fatal doses than were those of the controls, and in general the protected rats were less severely intoxicated following high non-lethal doses of bismuth. Recovery from ill effects was likewise noticeably more rapid in the protected groups. Protection was even more evident in the groups receiving bismuthyl mannonate and bismuthyl citrate than in the tartrate group.

In order to determine whether or not protection against pentavalent arsenicals and these preparations of bismuth could be due to a diuretic effect of the excess fluid injected with PABA, or perhaps the direct protection of the kidney, several trials were made using various dosages of sodium chloride solutions, and solutions of calcium lactate administered separately and in combination with sodium chloride. In no instance was there any positive indication of a protective action following such injections. Sections of kidney tissue also were prepared for microscopic study from a number of rats that had received carbarsone, some with and some without PABA. While varying degrees of damage could be observed in both the protected and unprotected rats, the damage, on the whole, was apparently as great in one group as in the other.

#### SUMMARY AND CONCLUSIONS

From the data presented herein it is concluded that p-Aminobenzoic acid exerts a definite but relatively low grade of protection of rats against the bismuth preparations, sodium bismuthyl citrate, sodium bismuthyl mannonate and sodium bismuthyl tartrate.

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# A PHARMACOLOGICAL EVALUATION OF DIHYDROERGOTAMINE METHANESULFONATE (D.H.E. 45)\*

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The clinical efficacy of dihydroergotamine methanesulfonate (D.H.E. 45—Sandoz) in the treatment of migraine headaches has been reported by Horton (1, 2), Hartman (3), Clein (4), Dannenberg (5) and others, while information regarding certain of its pharmacological actions has been presented by Rothlin (6), Brügger (7) and Kirchhof (8). The present report includes studies made during the past two years in a general pharmacological evaluation of the drug, particularly with reference to its acute and chronic toxic effects and the selective sympatholytic properties which it displays. A preliminary report of these studies has been made (9).

**METHODS.** The acute and chronic toxic effects of DHE were tested on albino rats of the Sprague-Dawley strain, on domesticated rabbits, and on puppies. The oxytocic action was determined on isolated, perfused guinea pig uteri and on rats during normal gestation. The possibility of electrocardiographic alterations were tested on monkeys (*Macacus rhesus*) and on dogs, and the latter species was used to test adrenolytic and sympatholytic properties during cyclopropane anesthesia and also during anesthesia with other agents when alterations of blood pressure *per se* or of response to epinephrine subsequent to administration of the ergot derivative were tried. A study of histological sections obtained from the kidneys, liver, spleen, heart and lungs of rats was made to ascertain possible pathological changes which might have been produced by the drug. Patients suffering from migraine headaches who previously had employed "Gynergen" advantageously compared such responses to this new therapeutic agent.

The drug was supplied in glass sealed ampules of one cc. delivery capacity in a concentration of 0.5 mg. or 1.0 mg. per cc., of which the latter strength was chiefly employed. Control series of animals were tested with ergotamine tartrate ("Gynergen") wherever such studies were indicated. The latter drug was available in ampules containing 0.5 mg./cc.

For tests of toxicity intravenous injections of DHE of 5, 10 and 20 mg./Kg. were made daily into rats for from two to forty times. Animals from each group served as the source of material for histological sections. Acute toxicity determinations by intravenous administration of the drugs also were attempted on rabbits using dosages as great as 35 mg./Kg. of DHE and 4.0 mg./Kg. of gynergen. Pairs of litter mate puppies six to ten weeks of age were injected subcutaneously daily with 0.25 or 0.5 mg./Kg. of gynergen or 0.5 or 1.0 mg./Kg. of DHE for periods of 15 to 100 days. Weights were determined at weekly intervals and the dosage adjusted for any increase which had occurred.

The activity of virgin guinea pig uteri in oxygenated baths of Locke-Ringer's solution in which gynergen or DHE could be added separately served as one method of determining

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oxytocic effects of the drug. Another test was the injection of virgin female rats for several days with gynergen or DHE, after which a male was put into each pen of four females and breeding permitted. The daily injections were continued in the females until the normal gestation period had been exceeded or litters were delivered. The mother with its litter then was put into an individual cage and growth rate of the young rats followed to adulthood.

The adrenolytic and sympatholytic properties of DHE were compared to previously evaluated effects of ergotamine tartrate (10) to prevent cyclopropane-epinephrine ventricular tachycardia which can be produced by a standard technique of administering epinephrine during such anesthetization (11). Electrocardiograms were taken throughout the entire period of injection of DHE. Doses of 0.05 to 0.4 mg./Kg. of the drug were diluted to a total volume of 5 cc. with normal saline solution and injected intravenously at a steady rate of 1 cc. each ten seconds. This volume of fluid and rate of injection were employed for all such intravenous procedures. The studies were extended to observations of the effects of the drug *per se* on monkeys and also of its protective value against cardiac irregularities which in the *Macacus rhesus* occur spontaneously, particularly in the induction phase of anesthesia.

Some of the dogs used in the tests with cyclopropane, as well as others anesthetized with diethyl ether, sodium ethyl (1-methyl butyl) barbiturate (nembutal-Na), sodium iso-amyl ethyl barbiturate (amytal), or chloroform, were prepared for direct determinations of blood pressure, as previously noted (12), so they could be tested on subsequent days with different dosages of the same drug or with other drugs. A recording of blood pressure was made during the injection of 0.4 mg./Kg. of DHE. Five minutes after the DHE injection 0.01 mg./Kg. of epinephrine was administered at a steady rate in the standard 50-second period and a second blood pressure tracing taken to determine whether an ergotamine-like reversal of the epinephrine pressor effect resulted.

**RESULTS.** Neither an acute nor a chronic toxic dosage of DHE could be determined for rats. Although four animals were killed immediately following the first or second injection of 10 mg./Kg. and one died suddenly following the twenty-fourth daily injection, 21 other animals received from 16 to 40 injections without any apparent ill effects. Since the greatest concentration in which DHE was furnished was the 0.1% solution, the administration of a 20 mg./Kg. dosage intravenously to rats represented a volume of fluid equivalent to 2% of the animal's weight. Such an amount would approximate the administration of 20 to 25% of the circulatory volume in the one to two minutes required for an injection. The definite increase which this produced in venous pressure was evident by a marked centrifugal flow of blood which occurred whenever pressure was lessened on the plunger of the syringe during the latter half of an injection. Such a dosage of 20 mg./Kg. was given to four animals for 40 days and no gross effect was apparent. Three animals from another group died promptly after 12 such injections but contamination of the drug was possible for this group. The first two or three injections of any dosage always produced generalized clonus, ruffled hair, increased secretions and stertorous breathing but subsequently no such signs or symptoms were evident. The results of the studies of chronic toxicity in 48 rats given injections of 5 mg./Kg. to 20 mg./Kg. for from two to forty days are summarized in table 1.

Also summarized in this table are the results of the effects of ergotamine tartrate given in dosages of 1.0 to 2.5 mg./Kg. It can be noted that gangrene developed at the tips of the tails and that there ultimately was sloughing of from



$\frac{1}{3}$  to  $\frac{1}{2}$  of the entire tail in *every* rat injected with gynergen. Animals receiving the lower dosages showed such damage much earlier than those injected with the 2.5

TABLE 1

*Results of tests of chronic toxicity of dihydroergotamine methanesulfonate (D.H.E. 45) and ergotamine tartrate (as gynergen) in rats*

DRUG INJECTED	DOSAGE	NO. OF ANIMALS	INTRAVENOUS ADMINISTRATIONS	RESULTS AND COMMENT
D.H.E. 45	5 mg./Kg.	4	22	No observable change in animals' actions after marked jitteriness following first 2 or 3 injections.
		2	32	No observable change in animals' actions after marked jitteriness following first 2 or 3 injections.
		5	33	One animal died suddenly 2-3 min. after 33rd day of injection.
		2	40	Sacrificed for histological sections.
	10	6	1-5	One killed at 1st; 3 at 2nd inj.
		3	16-20	No observable changes.
		5	21-25	One killed with 24th injection.
		4	26-30	
		6	33	
		2	35-40	Sacrificed for histological study.
	20	2	2	Sacrificed for sections 1 week later.
		3	12	All animals died suddenly. Questionable drug contamination.
		4	40	No observable gross changes. Minimal microscopic findings.
Gynergen	1.0	9	4 or 5	Necrosis and gangrene of tip of tail within 2 to 4 days.
		2	6-10	Necrosis and gangrene of tip of tail within 2 to 4 days.
		4	20-25	Pregnancy and delivery.
	1.25	3	2 or 3	Necrosis, gangrene, diarrhea noted in duration of treatment.
	2.5	3	5-10	Marked diarrhea. Gangrene of tip of tail after 7-10 days of injection.
Normal saline	2% of body weight	5	22	No observable changes. Microscopic areas of degeneration in heart comparable to treated animals.

mg. amount. Loewe and Lenke (13) explained this as being due to a marked diarrhea which ergotamine elicits in the animal since a greater excretion of the

drug occurs in those which receive larger dosages. The phenomenon of diarrhea and delay in appearance of gangrene was evident in the present study.

Five of seven rabbits receiving 4.0 mg./Kg. of ergotamine tartrate intravenously died within 24 hours while four animals given 30 mg./Kg. of DHE and three receiving 35 mg./Kg. survived.

The subcutaneously administered dosage of 0.5 mg./Kg. of DHE caused no noticeable effects on puppies, but twice that amount routinely elicited vomiting within 5 to 15 minutes in one animal. Gynergen administered in 0.25 or 0.5 mg./Kg. amounts elicited similar effects, i.e., the smaller dosage was innocuous while the larger amount regularly caused emesis. Vomiting was produced regularly in adult dogs when 0.4 mg./Kg. of DHE was given intravenously before anesthesia tests were made.

The puppies all continued to gain in weight. Those receiving either the small or large dosage of DHE gained at equivalent rates. The animals receiving the larger dosages of gynergen gained weight but only at a very low increment. These animals also had rough, dull, ruffled hair which was a distinct contrast to the sleek, glossy appearance of the other members of the litter. At adulthood the two animals which had been given the greater dosage of gynergen were not as large nor as heavy as their litter mates. Since but two animals were available for each dosage of the two drugs in these tests, the smallest litter mates conceivably could have been selected for the higher dosages of gynergen.

The isolated, perfused virgin guinea pig uterus subjected to DHE did not alter its rate or strength of activity but the addition of gynergen in the oxygenated solution was followed by an increase in tonus and in rate and strength of uterine contractions. This confirms previous observations (6) that DHE does not affect the uterus, i.e., that it lacks oxytocic action. Normal gestation and delivery occurred despite daily injections of either drug into normal rats. Treatment with ergotamine throughout the entire three week period was difficult due to the gangrene which it elicited in the animals' tails. Following administration of the two drugs there seemed to be a definite difference in the manner in which the mothers cared for their litters. Those which had received DHE raised most of the litter to maturity but the ergotamine treated mothers took little care of their offspring and the survival rate was but 25 to 33 per cent of the animals born. Treatment was always stopped upon delivery.

The electrocardiograms taken from dogs and monkeys during the entire 50-second period of injection of DHE, and at approximately 10-second intervals thereafter for 5 minutes, showed the same characteristic changes from normal for both species. Original sino-auricular tachycardias, averaging about 150 in the dog and 160-180 in monkeys, were slowed by 10 to 25 beats per minute. The only alteration of the electrical complex was a decrease by  $\frac{1}{2}$  to  $\frac{2}{3}$  in the amplitude of the T-wave. From an initial average negative potential of 3 or 4 mm. in lead II it dropped to 1 or 2 mm.

The results of studies of protection from cyclopropane-epinephrine ventricular tachycardia made on dogs are summarized in table 2. In previous tests (10) it was determined that 0.12 to 0.16 mg./Kg. of ergotamine tartrate afforded

protection which lasted for approximately two hours. From the table it is evident that in the present investigation such amounts of DHE were inadequate but that doses of 0.4 mg./Kg. gave protection to each of 8 animals tested. Subsequent administrations of epinephrine indicated in three animals that the protective action was present after approximately 30 minutes but not at 60 minutes, while in three other animals it lasted for two hours. Two additional animals had protection from ventricular tachycardia but the duration of the action was not determined.

TABLE 2

*Effects of dihydroergotamine methanesulfonate on cardiac irregularities produced deliberately in the dog or occurring spontaneously in the monkey*

NO. OF ANIMALS TESTED	AMOUNT OF D.H.E. 45 INJECTED	RESULTS
Dogs		
3	mg./Kg. .05 to .075	No protection from cyclopropane-epinephrine ventricular tachycardia in the dog.
8	0.2	3 animals still responded with ventricular tachycardia when 0.01 mg. of epinephrine per Kg. was injected. 5 animals were protected from such an arrhythmia.
8	0.4	All 8 of the animals in this group were protected from ventricular tachycardia. In 3 of the animals the protection lasted for more than the two hours over which tests were made. In 3 others protection was present for at least 27 and 35 minutes, respectively. The duration of protection was not determined in the other 2 animals.
Monkeys		
3	0.4	A-V nodal rhythm, ventricular extrasystoles and ventricular tachycardia occurred in all 3 animals in control anesthesia periods. On a subsequent day, the D.H.E. was injected i.v. 5 minutes before anesthetic induction with cyclopropane. The irregularities were entirely prevented in 2 of the monkeys and a marked reduction in their degree and duration occurred in the third animal.

When cyclopropane was administered to the *Macacus rhesus* each of three monkeys had severe cardiac irregularities which included auriculo-ventricular nodal block, ventricular extrasystoles and ventricular tachycardia. The irregularities were particularly evident during the induction stage of anesthesia. On a subsequent day 0.4 mg./Kg. of DHE was administered intravenously to the animals, and 5 minutes later anesthetic induction was begun. There was a complete absence of all irregularities in two of the monkeys and the third had a significant reduction in both the degree and duration of such irregularities as is indicated also in table 2.

In a previous report (9) it was stated, that with various anesthetic agents, the injection of epinephrine following DHE administration did not cause a typical ergotamine-type reversal of the blood pressure. An increase in the number of animals tested necessitates a modification of this statement in that such a failure of reversal invariably was not noted. Figure 1 shows two graphs of blood pressure representative of the results which were obtained in 9 of 12 such tests. When epinephrine was given after a single dosage of 0.4 mg./Kg. of DHE there was a rise but no subsequent fall of blood pressure below the initial level. As is evident from figure 2, an occasional animal had a fall of blood pressure after an

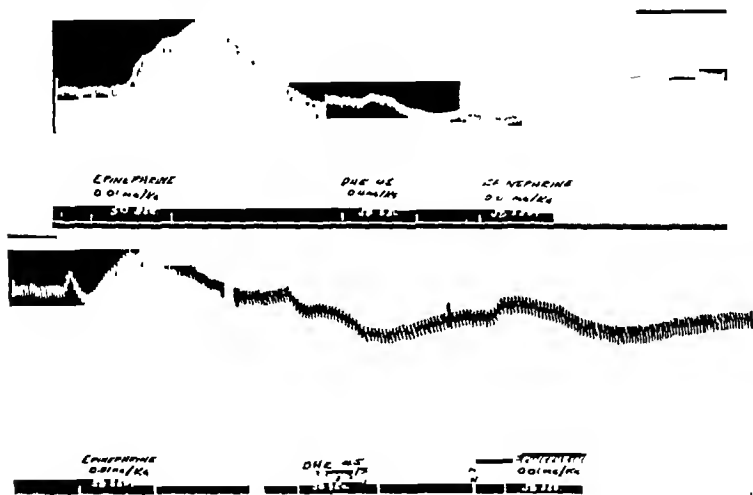


FIG. 1. GRAPHS OF BLOOD PRESSURES FROM TWO DOGS ANESTHETIZED WITH ETHER AND CHLOROFORM, RESPECTIVELY.

The responses to 0.01 mg./Kg. of epinephrine injected intravenously at a steady rate in 50 seconds both preceding and following 0.4 mg./Kg. of DHE 45 similarly administered are indicated. It is evident that a reversal in the blood pressure response was not produced by the ergot like alkaloid.

initial brief but definite rise in response to the epinephrine. There was at least a five minute lapse of time between administrations of any of the drugs. The total dosage of the ergot alkaloid was a factor since the administration of 0.2 mg./Kg. never caused a reversal to occur in the blood pressure when a subsequent injection of epinephrine was made. After a second 0.2 mg. Kg. dosage of DHE, an increase, then a decrease, in pressure followed in 3 of 6 animals when epinephrine was injected. The particular anesthetic agent was not a consistent factor in the type of response which occurred.

Twenty-one rats supplied tissue for histological sections which were taken from the tail, spleen, liver, kidney, heart and lung, fixed in 10% formalin, and stained with hematoxylin-eosin. Studies indicated that DHE caused no characteristic or significant changes in any of the organs. There were some minor changes such

as a generalized congestion in the kidneys of one of the animals which had received 10 mg./Kg. of the drug 36 times. In this group of animals, however, there was no cellular damage, necrosis or actual hemorrhage into renal tissue. Sections of the liver from the treated animals appeared to be better than those from an untreated group which served as controls. Some sections from the hearts of both treated and untreated animals had microscopic areas of scarring and fibrosis resulting from a slight focal destruction of fibers, but the myocardium itself was good. In the spleen and lung there was slight vascular congestion

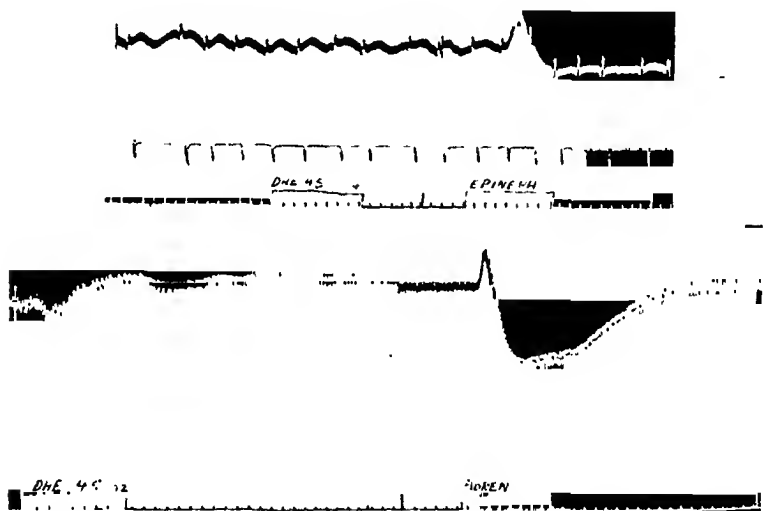


FIG. 2. GRAPHS OF BLOOD PRESSURES FROM TWO OTHER DOGS ANESTHETIZED WITH NEMBUTAL AND CYCLOPROPANE, RESPECTIVELY.

Epinephrine and DHE 45 were administered as described in fig. 1. It is evident that in both experiments there was a definite increase and subsequent decrease in blood pressure. The anesthetic agent used was not the responsible factor in the type of response of blood pressure. Respiration is recorded on the middle line of the upper graph. A previous 0.2 mg./Kg dosage of DHE had been administered to the second animal.

but no other pathological changes were evident. There was no significant difference in sections of tissue taken from animals treated with different dosages of the drug or injected a few or many times.

Six sufferers of typical migraine headache who previously had used gynergen successfully for relief of their attacks were supplied with DHE. Each patient voluntarily stated that equal or better results were obtained with the new compound in comparison with gynergen. The more favorable impressions were due to the absence of side effects from the DHE. Three stated that the side reactions which occur with gynergen are almost as annoying and time-consuming as the migrainous attack but that there were no such effects after DHE.

## SUMMARY AND CONCLUSIONS

Dihydroergotamine methanesulfonate (D.H.E. 45) must be administered in at least twice as great an amount as ergotamine tartrate (as "Gynergen") for comparable therapeutic results.

A solution containing 0.1% concentration of DHE does not permit administration of enough of the drug to make a determination of acute toxicity in Sprague-Dawley rats. Daily administrations of from five to twenty times as much DHE as gynergen were continued for two to three times as many days without the production of gangrene in the tails of any of the animals treated with DHE in contrast to the production of gangrene in the tails of every animal treated with gynergen.

Rabbits given intravenous injections of 4.0 mg./Kg. of gynergen usually were killed while none died with 30 and 35 mg./Kg. of DHE.

No oxytocic effects were produced by DHE either with *in vitro* tests of virgin guinea pig uteri or with *in vivo* tests of injections of the drug throughout the entire gestation period in albino rats.

Cardiac irregularities produced by the injection of epinephrine into dogs anesthetized with cyclopropane were completely inhibited by a preceding administration of 0.4 mg./Kg. of DHE. Epinephrine administrations which followed DHE injections generally did not produce a reversal of the blood pressure. With sufficiently large doses of DHE it could be elicited occasionally.

Cardiac irregularities produced spontaneously during the administration of anesthetic agents to monkeys were completely prevented by a premedicating dose of 0.4 mg./Kg. of DHE.

No significant pathological effects were evident in the sections of liver, kidney, spleen, heart or lung taken from rats after the administration of 5, 10, or 20 mg./Kg. doses of DHE even when injections were continued for 40 successive days.

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# ANOMALOUS DISTRIBUTION OF ANTIMONY IN WHITE RATS FOLLOWING THE ADMINISTRATION OF TARTAR EMETIC

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In the last few years, a number of studies have been conducted on the distribution of antimony in tissues following its parenteral administration. In these studies, blood levels have been of particular importance because of the proved value of antimonials in the treatment against parasites found in the blood such as the human schistosomes and *Diraofilaria immitis* of the dog. *Schistosoma mansoni* and *D. immitis* have been shown to exhibit a specific uptake of antimony (1, 2) presumably explaining, in part, the therapeutic effectiveness of antimonials. During a continuation of the latter studies, it was found that the blood concentration of antimony following a single dose of tartar emetic was similar in man and the dog (3).

The experiments reported in the present paper were carried out with the view of determining the pattern of distribution of antimony in the tissues of the albino rat, an animal which has been extensively used in pharmacologic studies.

**METHOD.** Radioantimony was prepared by the bombardment of an alloy of copper and antimony as a probe target in the 60-inch cyclotron of the Department of Terrestrial Magnetism, Carnegie Institution of Washington. Two isotopes of antimony were used,  $Sb^{121}$  with a half life of 60 days and  $Sb^{123}$  with a half life of 2.8 days.

The antimony was chemically separated from the other elements of the target as antimony trioxide and converted to tartar emetic. Blood and tissue samples were dried *in vacuo* over phosphoric acid anhydride at room temperature after careful wet weight measurements were made on each sample. The dry weights were then determined and the tissues ground to a uniform powder in a mortar. Standards were prepared by adding a known amount of tartar emetic containing the radioantimony to a weighed amount of tissue and dried exactly as the specimens containing the unknown amounts of antimony.

An aliquot of the dried tissue powder, usually 500 mgms., was spread evenly in a lucite cup, each sample or standard having a separate cup for measurement. The counts per unit time were determined by the use of a Geiger-Mueller tube with suitable sealing and counting circuits. By a direct comparison of the counts of the known standard and the unknown sample, after corrections were made for background radiation, sample size, weight lost in drying, and radioactive decay, the amount of antimony in the wet sample was determined.

**RESULTS.** All antimony was administered as tartar emetic. The concentration of antimony in the blood of dogs injected intravenously with 0.8 mgm. of antimony per kilogram of body weight is indicated in table 1. The blood level dropped very rapidly during the first 8 hours after injection. It will be noted that very small amounts of antimony remained in the blood after this interval,

the concentration remained at about 0.1 microgram during the following 3 days. In fact, other protocols indicate that amounts of about this magnitude remained in the blood for at least 14 days.

The blood concentrations were determined on a number of white rats after the intraperitoneal injection of 1.6 mgms. of antimony per kilogram of body weight as indicated in table 2. For the first 4 hours, these concentrations appear to parallel those of dogs. However at 8 hours the concentration is double that of

TABLE 1

*Micrograms of antimony per gram of blood of dogs after the intravenous administration of 0.8 mgm. of antimony per kilogram of body weight as tartar emetic*

HOURS AFTER INJECTION	NUMBER OF DOGS	AVERAGE MICROGRAMS OF ANTIMONY PER GRAM BLOOD
1	7	0.57
2	6	0.41
4	7	0.24
8	7	0.12
24	10	0.09
36	5	0.07
48	4	0.05
72	3	0.11

TABLE 2

*Micrograms of antimony per gram of blood of white rats after the intraperitoneal administration of 1.6 mgms. of antimony per kilogram of body weight as tartar emetic*

HOURS AFTER INJECTION	NUMBER OF WHITE RATS	AVERAGE MICROGRAMS OF ANTIMONY PER GRAM BLOOD
1	9	0.58
2	5	0.22
4	8	0.18
8	7	0.30
24	6	2.07
48	4	2.79
72	1	3.59

4 hours, and at 24, 48, and 72 hours there is a great increase in the concentration so that at this last interval the concentration has increased twentyfold over the 4-hour concentration.

In order to determine if the different dosages or routes of administration were responsible for the discrepancy in these concentrations, a dog was injected intraperitoneally with 1.6 mgms. of antimony per kilogram (the same dosage and route used for white rats). The results of the examinations of blood samples from this dog are indicated in table 3. It will be seen that the concentrations for the first 4 hours are higher than those of tables 1 and 2 but thereafter the curve parallels that of dogs injected with the lesser dosage. These blood concentrations are indicated in figure 1.



Data regarding the concentration of antimony in the liver, spleen, and kidneys are available for a few animals. Table 4 indicates these concentrations in dogs and table 5 indicates similar data for white rats. It will be observed that of the organs analyzed the liver invariably had the greatest concentration of antimony. It will be further noted that the livers of white rats contained only about half the concentration of antimony at 24, 48, and 72 hours as did those of dogs. No striking differences were observed in the antimony content of the spleen. The kidneys of rats appeared to have a much greater concentration of antimony at 24 and 48 hours than did those of dogs.

DISCUSSION. It is evident that a considerable difference occurs between dogs and white rats with respect to their blood-antimony concentrations. The recent work of Bartter et al. (3) indicates that the rate of disappearance of antimony from human blood after the administration of tartar emetic is almost identical

TABLE 3

*Micrograms of antimony per gram of blood of a dog after the intraperitoneal injection of 1.6 mgms. of antimony per kilogram of body weight as tartar emetic*

TIME AFTER INJECTION	MICROGRAMS ANTIMONY PER GRAM OF BLOOD
4 minutes	0.22
8 minutes	0.39
16 minutes	0.97
29 minutes	1.55
1 hour	1.52
2 hours	1.23
4 hours	0.77
8 hours	0.55
24 hours	0.33
36 hours	0.27
48 hours	0.28
72 hours	0.22

with that observed in dogs. Two graphs prepared from data of these authors are included in Figure 1 for comparison with graphs based on the data in our tables.

Incomplete data available for laboratory animals other than the dog indicate that the late high blood-antimony concentration in the white rat represents an anomalous situation. Pooled blood samples of groups of 5 white mice showed the following antimony concentrations per gram of blood after the intraperitoneal injection of 1.6 mgms. per kilogram: At 1 hour, 0.91 microgram; 24 hours, 0.09 microgram; 48 hours, 0.06 microgram; and at 72 hours, 0.07 microgram. In 6 cotton rats (*Sigmodon sigmodon hispidus*), the average blood concentration 24 hours after the intraperitoneal administration of 1.6 mgms. of antimony per kilogram was 0.22 microgram per gram. A guinea pig injected intraperitoneally with 1.6 mgms. of antimony per kilogram showed the following concentrations per gram of blood: At 15 minutes, 0.60 microgram; 1 hour, 1.11 micrograms; 2 hours, 0.94 microgram; 4 hours, 0.69 microgram; and at 8 hours, 0.47 microgram. Smith, Cowie, and Hill (1) have indicated graphically that the blood

antimony concentration of golden hamsters continued to decrease after the administration of tartar emetic when examinations were made at 1, 4, 12, 24, and 48 hours after injection.

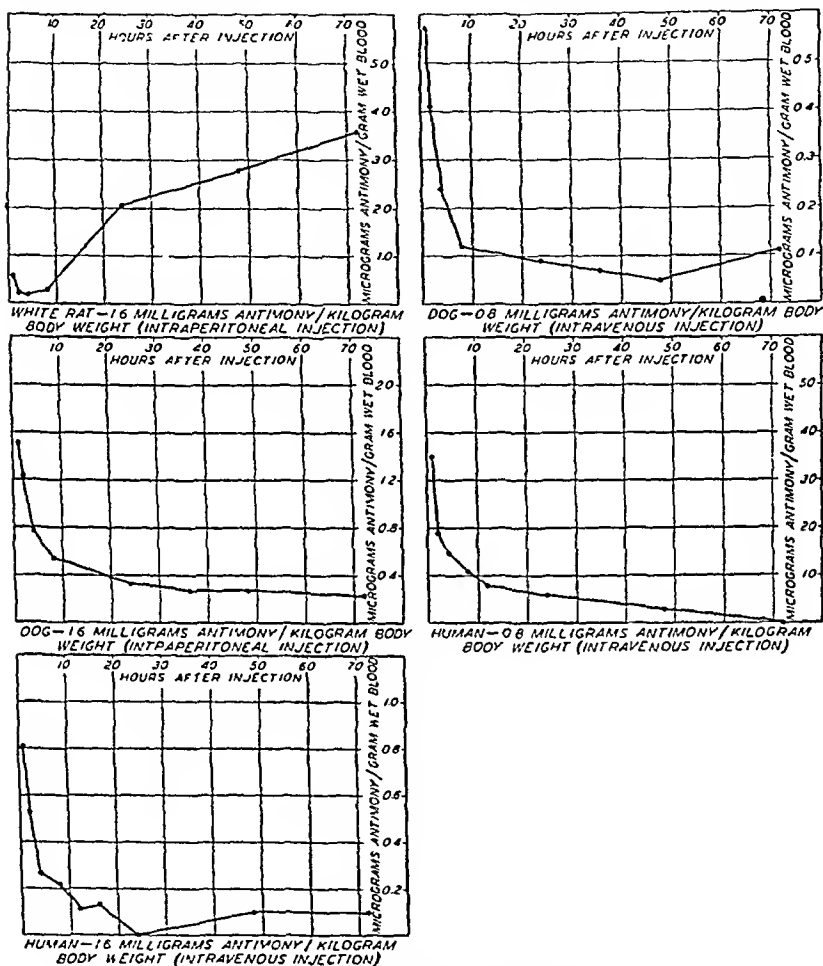


FIG. 1. GRAPHIC COMPARISON OF THE BLOOD CONCENTRATION OF ANTIMONY IN WHITE RATS, DOGS, AND MAN

Note that the ordinate scales are not uniform. The data for the curves in man are taken from Bartter et al. (3).

The blood samples of one dog were fractionated into the cellular constituents and plasma after the intravenous administration of 0.8 mgm. of antimony per kilogram. The results obtained on samples taken at various intervals up to 14

hours after injection are shown in table 6. The rate at which antimony leaves the plasma is even more rapid than the rate of its clearance from whole blood.

Of the tissues considered in this report, the livers of dogs showed a higher

TABLE 4

*Micrograms of antimony per gram of various tissues of dogs after the intravenous administration of 0.8 mgm. of antimony per kilogram of body weight as tartar emetic*

HOURS AFTER INJECTION	LIVER		SPLEEN		KIDNEY			
	No. dogs	Micrograms	No. dogs	Micrograms	Cortex		Medulla	
					No. dogs	Micrograms	No. dogs	Micrograms
24	4	9.79	4	0.50	4	0.78	3	0.33
36	5	11.10	4	0.54	2	0.80	2	0.33
48	1	7.25	1	0.60	1	0.72	0	—
72	1	6.39	1	0.99	1	0.60	0	—

TABLE 5

*Micrograms of antimony per gram of tissue of white rats after the intraperitoneal administration of 1.6 mgms. of antimony per kilogram of body weight as tartar emetic*

HOURS AFTER INJECTION	NO. RATS	LIVER	SPLEEN	WHOLE KIDNEY
2	1	6.90	0.46	2.78
4	1	16.70	0.38	2.36
8	1	10.77	0.52	3.39
24	1	4.13	0.55	3.22
48	4	2.18	1.33	1.11
72	1	2.66	0.45	0.65

TABLE 6

*Partition of antimony between plasma and blood cells during the first 4 hours after intravenous injection in a dog*

TIME AFTER INJECTION	MICROGRAMS ANTIMONY PER GRAM WHOLE BLOOD	MICROGRAMS ANTIMONY PER GRAM OF CELLS	MICROGRAMS ANTIMONY PER GRAM OF PLASMA	RATIO OF ANTIMONY IN CELLS AND PLASMA
15 minutes	1.3	1.8	0.25	7:1
30 minutes	1.1	2.0	0.23	9:1
1 hour	0.9	1.7	0.13	13:1
2 hours	0.7	1.2	0.06	20:1
4 hours	0.4	0.7	0.02	36:1

concentration of antimony 2 and 3 days after the injection than did those of white rats although the dogs received but half the dose given the white rats. The concentrations in the spleens of these animals appeared similar. At 24 and 48 hours, the kidneys of rats showed considerably more antimony per gram than did either the cortex or medulla of dog kidneys at the same intervals.

It appears from these observations that it is possible to infer something concerning the course of antimony through the tissues of the dog and rat. If we assume that the blood represents about 8 percent of the dog's weight, then after the intravenous injection of 0.8 mgm. per kilogram there would be 10 micrograms of antimony per gram of blood. The highest concentration found 15 minutes after the intravenous injection in any dog injected with this dosage was 1.3 micrograms indicating that most of the antimony had already left the blood. It would appear that most of this antimony had been absorbed by the liver in view of the large amounts recovered from that organ. While this process was going on, a considerable fraction of the antimony remaining in the blood was entering the erythrocytes from the plasma. The antimony in the liver must again reach the blood either by excretion in the bile and reabsorption by the intestine or by direct absorption from the liver. The available data do not indicate which method is of greater importance.

In the case of the rat, the highest concentration in the liver was found 4 hours after injection. The amount decreased thereafter. The concentration in whole kidneys was highest in the 8 and 24 hour samples and decreased thereafter. It would appear that in the rat antimony accumulates in the liver during the interval in which the blood concentration is the lowest, and is then discharged into the blood for the next several days. In spite of an increasing blood concentration after the first day, the kidney concentration showed a gradual decrease.

The quantitative determination of antimony by the use of radioactivity gives no clue as to the chemical state of the antimony. Indirect evidence indicates that the antimony compound accounting for the high blood concentrations of 2 and 3 micrograms per gram of blood in white rats is considerably less toxic than the originally injected tartar emetic. None of the rats showed evidence of antimony intoxication during these experiments. On the other hand, doses sufficient to cause blood concentrations as high as 2 micrograms per gram of blood in dogs were fatal. These concentrations were observed only within the first hour after injection.

The white rat has been used to a considerable extent in screening antimony compounds for their therapeutic possibilities. In view of the discrepancy between blood levels in this animal and in man, the results of screening should be interpreted with caution until it is shown that the compound accounting for the antimony in the blood at later intervals is not therapeutically effective.

#### SUMMARY

Dogs and rats were injected with tartar emetic prepared from radioactive antimony. Antimony concentrations in the blood and tissues of rats were considerably different from those in dogs and, judged by available data, were also different from concentrations encountered in man, white mice, cotton rats, and hamsters.

In white rats, the blood antimony is characterized by an increasing concentration beginning at 8 hours and lasting at least for 72 hours. This is in con-

trast to that in man and in the dog in which the concentration is decreasing to very low levels at these intervals.

There is some indication that the antimony compound appearing in the blood of the white rat at later intervals is considerably less toxic to the rat than its tartar emetic precursor. It is suggested that results obtained in screening antimonial compounds in white rats be interpreted with caution until more is learned of the phenomenon.

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# STUDIES ON THE LOCAL ANESTHETIC PROPERTIES OF 131 ALKYLAMINO ALCOHOL ESTERS

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With the technical assistance of CHARLES F. BUTZ

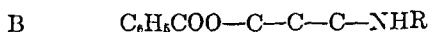
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In a series of papers (1, 2, 3, 4) dealing with the synthesis of alkylamino alcohols and their esters, Arthur C. Cope, Evelyn M. Hancock, et al. described the preparation of a large series of compounds having the basic type structure:



or



These authors included in their publications brief summaries dealing with the toxicities and local anesthetic properties of these compounds. More complete pharmacological data on these substances are presented in this communication.

The compounds were first screened on the basis of solubility. The 131 more readily soluble preparations were next investigated for toxicity following subcutaneous injection for surface anesthesia and irritation, and for infiltration anesthetic properties. Cocaine and procaine were used as reference standards.

The object of this preliminary investigation was to eliminate those samples which did not compare favorably with the reference standards. Since a relatively small number of animals were used in these tests, the results present only a rough quantitative estimate of their activity. Those compounds which compared favorably with the reference standards were tested for intravenous toxicity, for irritation following intradermal injection and for stability to autoclaving in aqueous solution. Finally, a certain few compounds found to be equal or superior to cocaine and procaine were submitted to a more extensive pharmacological study.

**METHODS** *Toxicity* Mice from the Carworth strain, having an average weight of 20 grams were used for these tests. For the determination of subcutaneous toxicity, at least 5 mice were used per dose level, to determine intravenous toxicity, 10 mice were used per dose level.

*Surface anesthesia* The method used was similar to that as described by Sollmann (5). A heavy bristle was substituted for the lead pencil in testing the anesthetization of the eye of rabbits. The bristle was brushed across the eye at least twice for each determination, care being taken to approach along the side of the animal's head so that a visual reaction would not occur. The pouch formed by pulling the lower lid was filled with the test solution which remained in contact with the eye for 2 minutes, after which time any excess was allowed to escape. The sensitivity of the cornea was determined at 5 minute intervals until the blinking reflex reappeared. Since there was considerable variation in sensitivity among individual rabbits, the drug to be tested was applied to one eye in concentrations

ranging from 0.1 to 1% and cocaine in 0.25% solution was applied to the other eye. The efficacy of the preparation is expressed as the quotient of the duration of anesthesia in minutes divided by the per cent concentration used and also by comparing its efficacy with that of cocaine as determined in the same animal. Two rabbits were used in testing each compound except samples which seemed particularly interesting which were tested on as many as 12 rabbits.

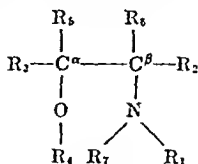
*Surface irritation.* Observations for signs of irritation were made immediately after application of the anesthetic to the eye and at 1, 24 and 48 hours after the test. Additional irritation tests were conducted with weaker or more concentrated solutions to determine the approximate minimal concentration necessary to produce irritation of the cornea or conjunctiva.

*Infiltration anesthesia.* The method of Molitor and Robinson (6) was employed. The guinea pigs were first sensitized with 35 mg./kg. 'Pernoston' hypnotic injected subcutaneously. The abdomen was shaved and 0.1 cc. of 0.1%, 0.25% and 0.5% solutions of the drug to be studied was injected subcutaneously. A like amount of similar concentrations of procaine was injected in comparable areas of the same guinea pig to serve as controls. A uniform painful stimulus, produced by forceps adjusted so that a constant pressure was applied to the infiltrated area, and the time required for the return of the response to this stimulus noted. The calculations of efficacy were made in the same manner as those for surface anesthesia.

*Intradermal irritation.* The compounds were tested for irritation by injecting intradermally 0.1 cc. of 0.1%, 0.25% and 0.5% solution of each compound into the depilated abdominal skin of guinea pigs (and into the skin on the arms of human subjects). Comparable areas in the same animals were injected with a like volume of similar concentrations of procaine. A comparison of the degree of irritation resulting from the injection of the drug with that produced by procaine was made using the areas of erythema, edema and tissue damage as criteria.

*Stability.* Solutions were autoclaved for 20 minutes at 15 pounds pressure and then tested for topical anesthesia. Compounds with the same anesthetic activity after autoclaving as before were regarded as stable.

*Substances studied.* The various compounds have been tabulated in 15 different classes. Fourteen of these classes can be represented by a structural key:



$R_1$  = N-alkyl (substituent) or (group) as indicated above in skeletal formula.

$R_2$  =  $\beta$ -alkyl groups

$R_3$  =  $\alpha$ -alkyl substituents

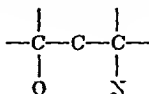
$R_4$  = Acyl group (of ester); benzoyl or p-aminobenzoyl

$R_5$  =  $\alpha$ -alkyl substituent

$R_6$  =  $\beta$ -alkyl groups

$R_7$  = Same as  $R_1$ —N-alkyl (substituent) or (group); usually hydrogen.

The remaining class has the following skeletal structure common to all members:



- Group 1 Benzoate HCl of 2-alkylaminoethanol  
 $C_6H_5COOCH_2CH_2NHR \cdot HCl$   
 $R_1$  varies  
 $R_4 = C_6H_5CO$   
 $R_2, R_3, R_5, R_7 = H$
- Group 1A p-aminobenzoate HCl of 2-alkylaminoethanol  
 Otherwise the same as Group 1.
- Group 2 Benzoate HCl of 1-alkylamino-2-propanols  
 $C_6H_5COOCH(CH_3)CH_2NHR \cdot HCl$   
 Same as Group 1 except  $R_2$  is methyl ( $CH_3$ ) instead of ( $H$ ).
- Group 2A p-aminobenzoate HCl of 1-alkylamino-2-propanols  
 Otherwise same as Group 2.
- Group 3 Benzoate HCl of 2-alkylamino-1-propanol  
 $C_6H_5COOCH_2CH(CH_3)NHR \cdot HCl$   
 Same as Group 1 except  $R_2$  is methyl  
 Same as Group 2 except methyl is on  $R_2$  instead of  $R_1$ .
- Group 3A p-aminobenzoate HCl of 2-alkylamino-1-propanol  
 Otherwise same as Group 3.
- Group 4 Benzoate HCl of 2-alkylamino-1-butanol  
 $C_6H_5COOCH_2CH(C_2H_5)NHR \cdot HCl$   
 Same as Group 1 except  $R_2$  is ethyl  
 Same as Group 3 except  $R_2$  is ethyl instead of methyl.
- Group 4A p-aminobenzoate HCl of 2-alkylamino-1-butanol  
 Otherwise same as Group 4.
- Group 5 p-aminobenzoate HCl of 2-alkylamino-2-methyl-1-propanol.  $p-NH_2C_6H_4-$   
 $COOCH_2C(CH_3)_2NHR \cdot HCl$   
 Same as Group 1A except  $R_2$  and  $R_3$  are methyl.  
 Same as Group 3A except  $R_4$  (as well as  $R_2$ ) is methyl.
- Group 6 Benzoate HCl of 1-alkylamino-2-methyl-2-propanol  
 $C_6H_5COOC(CH_3)_2CH_2NHR \cdot HCl$   
 Same as Group 1 except  $R_2$  and  $R_3$  are methyl.  
 Same as Group 2 except  $R_1$  (as well as  $R_2$ ) is methyl.
- Group 6A p-aminobenzoate of 1-alkylamino-2-methyl-2-propanol.  
 Otherwise the same as Group 6.
- Group 7 Esters of 1-cyclohexylamino-2-methyl-2-propanols  
 $RCOOC(CH_3)_2CH_2NHC_6H_{11} \cdot HCl$   
 $R_4$  varies-esters of various acids  
 $R_2$  and  $R_3$  are methyl ( $CH_3$ )  
 $R_2, R_4, R_7$  are (II)  
 $R_1$  is cyclohexyl
- Group 8 Does not fit in formula.  
 p-aminobenzoate HCl of 3-alkylamino-1-propanols  
 $p-NH_2C_6H_4COOCH_2CH_2CH_2NHR \cdot HCl$   
 These compounds have three carbon atoms between the ester and amino group  
 instead of two as in Group 1.  
 $R_4 = p\text{-aminobenzoyl}$   
 $R_1$  varies



- Group 9 p-aminobenzoates of 2-dialkylamino ethanol  
 $p\text{-NH}_2\text{C}_6\text{H}_4\text{COOCH}_2\text{CH}_2\text{NR}_2\cdot\text{HCl}$   
 $\text{R}_1 = p\text{-aminobenzoyl}$   
 $\text{R}_2$  and  $\text{R}_3$  vary
- Group 10 Same as Group 9 except  $\text{R}_2$  is methyl
- Group 11 p-dialkylaminobenzoate HCl of 2-alkylamino ethanol  
 $p\text{-R}_2\text{NC}_6\text{H}_4\text{COOCH}_2\text{CH}_2\text{NHR}\cdot\text{HCl}$   
 $\text{R}_1 = p\text{-dialkylaminobenzoyl}$   
 $\text{R}_2$  and  $\text{R}_3$  vary
- Group 12 Same as Group 11 except  $\text{R}_2 = \text{methyl}$
- Group 13 Same as Group 11 except  $\text{R}_2 = \text{ethyl}$
- Group 14 Phenyl urethane HCl of 2-alkylamino ethanol  
 $\text{C}_6\text{H}_5\text{NHCOOCH}_2\text{CH}_2\text{NHR}\cdot\text{HCl}$   
 $\text{R}_1 = \text{phenyl urethane}$   
 Otherwise same as Group 1.
- Group 15 Same as Group 14 except  $\text{R}_2 = \text{methyl}$

RESULTS. The quantitative results of the screening tests of the more interesting compounds are presented in table 1. This table does not contain all of the compounds tested. Many have been omitted as they already appear in print (1, 2, 3, 4) and the additional information that was obtained is of no particular value as these compounds were found to be too toxic, too irritating or lacking anesthetic activity.

For purposes of selecting compounds for further pharmacological study and possible clinical use, the following criteria were used: a topical and infiltration anesthetic index equal to or greater than 1, irritation comparable to or less than cocaine or procaine, good solubility and stability to autoclaving. Of the 9 compounds (marked with an asterisk \*) which were found to compare very favorably with both procaine and cocaine, three, namely, 2-cyclopentylamino-1-propyl-p-aminobenzoate HCl, 1-Cyclopentylamino-2-propylbenzoate HCl, 2-isopropylamino-1-butylbenzoate HCl, were selected for further pharmacological investigation (table 2). 2-Isopropylamino-1-butylbenzoate HCl was the least irritating on topical application, possessed a low intravenous toxicity and was readily soluble. This compound showed a slight loss of anesthetic potency on autoclaving, which however, did not significantly reduce its higher anesthetic index in comparison to procaine or cocaine. 1-Cyclopentylamino-2-propylbenzoate HCl had the best infiltration anesthetic index and its solubility and stability were satisfactory. 2-Cyclopentylamino-1-propyl-p-aminobenzoate HCl produced no topical irritation at 5% concentration, was no more irritating on intradermal injection than procaine, was readily soluble and was stable to autoclaving.

1-Cyclopentylamino-2-propylbenzoate HCl compared favorably to procaine and cocaine on the basis of the anesthetic index computed from the subcutaneous toxicity. However, on the basis of the anesthetic index computed from the intravenous toxicity this compound has a higher anesthetic index than cocaine but possesses no advantage over procaine. This compound causes mydriasis

TABLE 1

ALKYL GROUP, R <sub>1</sub>	TOXICITY—MICE		ANESTHETIC EFFICIENCY		IRRITATION	
	L.D. 50 (mg./kg.)		Surface Cocaine = 1	Infiltration Procaine = 1	Cornea of rabbit	Intradermal guinea pig
	S.C.	I.V.				
Group 1A—p-aminobenzoate HCl of 2-alkylaminoethanol, p-NH <sub>2</sub> C <sub>6</sub> H <sub>4</sub> COOCH <sub>2</sub> CH <sub>2</sub> NHR·HCl R <sub>1</sub> = p-aminobenzoyl      Otherwise the same as group 1						
3-Pentyl	425		1	1	Slight at 1.0%	Irritating at 0.5% Slight at 0.025%
2-(4-Methylpentyl)	125		1	1	None at 0.5%	
2-Heptyl	125		2	2	None at 0.5%	
2-Octyl	250	33	5	5	None at 1.0%	
2-Nonyl	200		2	1	Irritating at 0.5%	Irritating at 1%
5-Nonyl	175		1	1	Irritating at 1.0%	
2-Decyl	250		2	1	Irritating at 0.5%	
Cyclohexyl	400		1	2	Slight at 1.0%	
3,3,5-Trimethyl-cyclohexyl	225		1	1.5	Slight at 0.5%	
Butyl	250		0.2	1.5	None at 5.0%	
Isobutyl	450		0.5	2	None at 1.0%	
n-Amyl	125		1		None at 0.5%	
2-Octyl sulfate	175		2	1.5	Slight at 0.5%	
2-Octyl sulfamate	175		1	2	Slight at 0.25%	
2-Octyl d-tartrate	200		0.7	2	None at 1.0%	
2-Octyl glycolate	200		2.5	2.5	Slight at 1.5%	
2-Octyl citrate	150		2.5		Slight at 0.1%	
Group 2—Benzoate HCl of 1-alkylamino-2-propanols, C <sub>6</sub> H <sub>5</sub> COOCH(CH <sub>3</sub> )CH <sub>2</sub> NHR·HCl R <sub>1</sub> varies      R <sub>2</sub> = methyl (CH <sub>3</sub> )      R <sub>3</sub> = C <sub>6</sub> H <sub>5</sub> CO      R <sub>4</sub> , R <sub>5</sub> , R <sub>6</sub> = H						
Cyclopentyl*	1000	35	0.75	5	Slight at 2.0% None at 1.0%	= to procaine
Cyclohexyl*	600	30-35	0.75	2-2.5	Slight at 2.0% None at 1.0%	= to procaine

TABLE 1—Continued

ALKYL GROUP, R <sub>1</sub>	TOXICITY—MICE		ANESTHETIC EFFICIENCY		IRRITATION	
	L.D. 50 (mg./kg.)		Surface Cocaine = 1	Infiltration Procaine = 1	Cornea of rabbit	Intradermal guinea pig
	S.C.	I.V.				
Group 2A—p-aminobenzoate HCl of 1-alkylamino-2-propanols, p-NH <sub>2</sub> C <sub>6</sub> H <sub>4</sub> COOCH(CH <sub>3</sub> )CH <sub>2</sub> NHR·HCl R <sub>1</sub> = p-aminobenzoyl      Otherwise the same as group 2						
5-Nonyl	225		2.5	2	Slight at 0.25% None at 0.1%	Irritating at 0.05%
4-(2,6-Dimethylheptyl)	250	35	2.5	2	Slight at 0.5%	Irritating at 0.25% Slight at 0.05%
4-Glycolate	475	35	5	5	Slight at 0.5%	Irritating at 0.25% Slight at 0.05%
5-(2,8-Dimethylnonyl)	>400		2	3	Irritating at 0.1%	Slight at 0.05% Irritating at 1%
Cyclohexyl	250	25	1	4	None at 2.0%	Slight at 0.025%
Cyclohexyl glycolate	250		2.5	2.5	None at 1%	Slight at 0.05%
Cyclopentyl*	400	40	2	2	None at 3.0%	= to procaine
Group 3—Benzoate HCl of 2-alkylamino-1-propanol, C <sub>6</sub> H <sub>5</sub> COOCH <sub>2</sub> CH(CH <sub>3</sub> )NHR·HCl R <sub>1</sub> varies      R <sub>2</sub> = methyl (CH <sub>3</sub> )      R <sub>3</sub> = C <sub>6</sub> H <sub>5</sub> CO      R <sub>4</sub> , R <sub>5</sub> , R <sub>7</sub> = H						
Cyclohexyl*	1000	35	2.5	2-3	Slight at 1.0% None at 0.5%	= to procaine
Cyclopentyl*	>1000	50	2.5	2	Slight at 3.0% None at 2.0%	None at 0.5%
4-(2,6-Dimethylheptyl)	>1000		2.5	2	Slight at 1.0% None at 0.5%	Irritating at 0.1%
Group 3A—p-aminobenzoate HCl of 2-alkylamino-1-propanol, p-NH <sub>2</sub> C <sub>6</sub> H <sub>4</sub> COOCH <sub>2</sub> CH(CH <sub>3</sub> )NHR·HCl R <sub>1</sub> = p-aminobenzoyl      Otherwise the same as group 3						
3-Pentyl	550		1	2	None at 3%	Very slight at 0.05%
4-Heptyl	350		2.5	2	Slight at 2% None at 1%	Slight at 0.05%
5-Nonyl	250		2.5	2	Slight at 0.25% None at 0.1%	Irritating at 0.05%
4-(2,6-Dimethylheptyl)	600		3	2	Slight at 0.5% None at 0.25%	Slight at 0.025%
Cyclohexyl	550		2	4	None at 1.2%	Slight at 0.025%
Cyclopentyl*	750	35	2-2.5	2	None at 5%	None at 0.5%

TABLE 1—Continued

ALKYL GROUP, R <sub>1</sub>	TOXICITY—MICE		ANESTHETIC EFFICIENCY		IRRITATION	
	L.D. 50 (mg./kg.)		Surface Cocaine = 1	Infiltration Procaine = 1	Cornea of rabbit	Intradermal guinea pig
	S.C.	I.V.				
Group 4—Benzoate HCl of 2-alkylamino-1-butanol, C <sub>6</sub> H <sub>5</sub> COOCH <sub>2</sub> CH(C <sub>2</sub> H <sub>5</sub> )NHR·HCl						
R <sub>1</sub> varies	R <sub>2</sub> = ethyl		R <sub>4</sub> = C <sub>6</sub> H <sub>5</sub> CO		R <sub>3</sub> , R <sub>5</sub> , R <sub>7</sub> = H	
Cyclohexyl*	>750	25	2.5	2.5	None at 1.5%	= to procaine
Isopropyl*	>1000	60	0.75	2-2.5	None at 10.0%	None at 0.5%
Cyclopentyl	750	30	0.5	3-5	None at 1.5%	= to procaine
Group 4A—p-aminobenzoate HCl of 2-alkylamino-1-butanol, p-NH <sub>2</sub> C <sub>6</sub> H <sub>4</sub> COOCH <sub>2</sub> CH(C <sub>2</sub> H <sub>5</sub> )NHR·HCl						
R <sub>1</sub> = p-aminobenzoyl			Otherwise the same as group 4			
Isopropyl	700		2-2.5	2-2.5	None at 1.5% Slight at 2.0%	= to procaine
3-Pentyl	550	32	1	1	Very slight at 1%	
4-Heptyl	250	30	2.5	2.5	Slight at 0.25%	Very slight at 0.05%
4-(2,6-Dimethylheptyl)	700	40	10	3	Irritating at 0.25%	Severe at 0.25% Slight at 0.025%
4-(2,6-Dimethylheptyl-Glycolate)	700	40	5	4		Slight at 0.25%
Cyclohexyl	500	30	2	2.5	Slight at 1%	Slight at 0.25%
Cyclohexyl glycolate	400	30	2	3	Slight at 1%	Slight at 0.25%
Cyclopentyl*	750	35	2	2	None at 1.5%	= to procaine
Group 5—p-aminobenzoate HCl of 2-alkylamino-2-methyl-1-propanol, p-NH <sub>2</sub> C <sub>6</sub> H <sub>4</sub> COOCH <sub>2</sub> C(CH <sub>3</sub> ) <sub>2</sub> NHR·HCl						
R <sub>1</sub> varies	R <sub>2</sub> , R <sub>5</sub> = methyl (CH <sub>3</sub> )		R <sub>4</sub> = p-aminobenzoyl		R <sub>3</sub> , R <sub>6</sub> , R <sub>7</sub> = H	
Cyclohexyl	225		2.5	4	None at 0.6%	
Group 6—Benzoate HCl of 1-alkylamino-2-methyl-2-propanol, C <sub>6</sub> H <sub>5</sub> COOC(CH <sub>3</sub> ) <sub>2</sub> CH <sub>2</sub> NHR·HCl						
R <sub>1</sub> varies	R <sub>2</sub> , R <sub>5</sub> = methyl (CH <sub>3</sub> )		R <sub>4</sub> = C <sub>6</sub> H <sub>5</sub> CO		R <sub>3</sub> , R <sub>7</sub> = H	
Cyclopentyl	200	25	2-2.5	2.5	Slight at 2 % None at 1.5%	= to procaine
Group 6A—p-aminobenzoate of 1-alkylamino-2-methyl-2-propanol, p-NH <sub>2</sub> C <sub>6</sub> H <sub>4</sub> COOC(CH <sub>3</sub> ) <sub>2</sub> CH <sub>2</sub> NHR·HCl						
R <sub>1</sub> = p-aminobenzoyl			Otherwise the same as group 6			
2-Heptyl	100		4	10	None at 1%	Slight at 0.1% Severe at 0.5%
2-Octyl	100		4	10	Irritating at 1%	Severe at 0.5% Slight at 0.1%
Cyclohexyl	100	15	4	10	Slight at 1%	None at 0.5%
Cyclopentyl	75	35	2-2.5	3-4	None at 5%	None at 0.5%
Isoamyl	35		2	5	Slight at 1%	= to procaine

TABLE 1—*Concluded*

ALKYL GROUP		TOXICITY—MICE		ANESTHETIC EFFICIENCY		IRRITATION	
R <sub>1</sub>	R <sub>2</sub>	L.D. 50 (mg./kg.)		Surface Cocaine = 1	Infiltration Procaine = 1	Cornea of rabbit	Intradermal guinea pig
		S.C.	I.V.				
Group 9—p-aminobenzoate HCl of 2-dialkylamino ethanols, p-NH <sub>2</sub> C <sub>6</sub> H <sub>4</sub> COOCH <sub>2</sub> CH <sub>2</sub> NR <sub>2</sub> ·HCl							
R <sub>1</sub> = p-aminobenzoyl		R <sub>1</sub> and R <sub>2</sub> vary		R <sub>2</sub> , R <sub>3</sub> , R <sub>4</sub> , R <sub>5</sub> = H			
2-Octyl	Methyl	300		2.5	2	None at 2%	Irritating at 0.05%
2-Octyl	Ethyl	300		2.5	2	Irritating at 0.1%	Slight at 0.1% None at 0.05%
Cyclohexyl	Methyl	300		1	4	Slight at 1%	None at 0.5%
Cyclohexyl	Ethyl	450		2	2	Slight at 0.1%	Slight at 0.1% None at 0.05%

as does cocaine and has the added disadvantage in that it produces a slight depression in blood pressure on intravenous injection.

2-Cyclopentylamino-1-propyl-p-aminobenzoate HCl and 2-isopropylamino-1-butylbenzoate HCl were found to produce the same degree of irritation and to effect the circulatory and respiratory systems in the same degree as does procaine. Both compounds produce a longer duration of anesthesia than procaine or cocaine and have a higher anesthetic index than procaine and cocaine as computed from either the subcutaneous or intravenous toxicity. These compounds may be regarded as both infiltration and surface anesthetics. However, 2-cyclopentylamino-1-propyl-p-aminobenzoate HCl has several advantages in that it is completely stable, does not produce mydriasis and may be used to advantage either as an infiltration anesthetic or to produce surface anesthesia.

DISCUSSION. Although the relation between chemical structure and pharmacological activity of the 131 compounds investigated cannot be evaluated definitely, the following general tendencies are noted.

1. In p-aminobenzoates, substitutions of the cyclohexyl, cyclopentyl and 4(2,5-dimethyl heptyl) groups as the R<sub>1</sub> substituent enhance the anesthetic activity regardless of other substitutions.

2. Benzoate and p-aminobenzoate esters containing  $\beta$ -methyl or  $\beta$ -ethyl substituents decrease the subcutaneous toxicity in mice.

3.  $\alpha$ -Amino esters show increased subcutaneous toxicity in mice, increased irritation and decreased solubility.

4. A comparison of the molecular weights of the compounds in each group to the anesthetic potency did not as a rule indicate increased anesthetic potency with an increase in molecular weight.

While most of the comparisons and evaluations of these compounds are derived from animal studies, there appears to be a definite parallelism between the results obtained with animals and those observed in a limited number of experiments with humans.

TABLE 2

COMPOUND	TOXICITY IN MICE		ANESTHETIC EFFICIENCY		IRRITATION			GENERAL ANESTHETIC CONCENTRATION			IRRITATION		DURATION OF ANESTHETIC INTRADERMAL IN HUMAN ARM		CIRCULATION AND RESPIRATION		SYNCHRONISTIC ACTION WITH ADRENALIN 1:50,000	STIMULUS HEART	MUSCLE NERVE REF. PROGS.	PERFUSION PROGS. LEGS
	L.D. 50 (mg./kg.) S.C.	L.D. 50 (mg./kg.) I.V.	Topical rabbit eye	Intra-dermal rabbit eye	Topical rabbit eye	Intra-dermal rabbit eye	Intra-dermal human skin	Stim. HRT	Topical	Intra-dermal	Pupill. dilation	1% sol'n repeated daily for 5 days	1% opth. sol'n repeated daily for 6 days	0.5% 20 min	1.0% 3-4 hrs	4 mg./kg. cat blood pres. sure	4 mg./kg. cat respi- ration			
2-(3,5-dibenzylamino-1-propyl) p-amino-benzoate HCl	750-1000	40	2-2.5	3.5	5%	0.5%	+	+	0.03% 0.05%	-	-	-	-	0.5% 20 min	1.0% 3-4 hrs	+	(4) +	+	+	1
1-Cy3,5-dibenzylamino-2-propyl benzoate HCl	>1000	35	1	5	2% + - 1%	0.5% + -	-	+	0.1% 0.05%	+	+	-	-	35 min	3-4 hrs	- (3)	++	+	++	1
2-Isopropyl-amino-1-hetyl benzoate HCl	>1000	50	0.75	2.5	10%	0.5% + -	-	+	0.1% 0.05%	+	+	-	-	120 min	3-4 hrs	+	+	+	+	1
Procaine	650	85	1	1	-	0.5% + - (1)	+	+	0.1%	-	-	-	-	30-40 min.	2 hrs.	+	+	++	++	1
Cocaine	150	30-35	1	-	2% + - 1%	-	-	-	0.1%	+	(2)	-	-	-	-	+++	+++	+	++	-

(1) Burning sensation directly after injection of procaine.

(2) A 10% solution caused a dilation in rabbit's eyes from a normal diameter of 5 mm. to 7.5 mm.

(3) Caused drop in blood pressure—other compounds produced an elevation of blood pressure.

(4) + Slight depression; ++ respiration stopped for short interval; +++ respiration stopped for long interval

(5) + = dilution of 1:50,000 required to stop heart; ++ = dilution of 1:5,000 required to stop heart.

## CONCLUSIONS

One hundred and thirty-one compounds have been tested for surface and infiltration anesthesia, toxicity, irritation and stability. Nine of these compounds (1-cyclopentylamino-2-propyl-p-aminobenzoate HCl, 1-cyclopentylamino-2-propyl benzoate HCl, 1-cyclohexylamino-2-propyl benzoate HCl, 2-cyclopentylamino-1-propyl p-aminobenzoate HCl, 2-cyclohexylamino-1-propyl benzoate HCl, 2-cyclopentylamino-1-propyl benzoate HCl, 2-cyclopentylamino-1-butyl p-aminobenzoate HCl, 2-cyclohexylamino-1-butyl benzoate HCl, 2-isopropylamino-1-butyl benzoate HCl) were found to have desirable surface and infiltration anesthetic properties.

Compounds 2-cyclopentylamino-1-propyl p-aminobenzoate HCl and 2-isopropylamino-1-butyl benzoate HCl were found to be comparable to procaine and superior to cocaine.

Compound 2-cyclopentylamino-1-propyl p-aminobenzoate HCl appears to be adaptable for surface as well as infiltration anesthesia.

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THE RELATIVE VALUE OF SYNTHETIC QUINIDINE, DIHYDRO-  
QUINIDINE, COMMERCIAL QUINIDINE, AND QUININE IN  
THE CONTROL OF CARDIAC ARRHYTHMIAS\*

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Early in the war between the United States of America and Japan (1941-1945) it became apparent that a shortage of the supply of quinidine sulphate for cardiac therapy would result from the Japanese seizure of the main source of the world's supply on the island of Java. Some, but at that time inadequate, amounts could still be obtained from the original but neglected stands of cinchona trees in South America. Hence it became of importance to determine whether the hitherto commonly employed commercial quinidine sulphate could be replaced effectively either by quinine salts, the supply of which was more ample, or by pure ("synthetic") quinidine or dihydroquinidine made therefrom. This problem was presented to, and actively taken up by, the Subcommittee on Cardiovascular Diseases of the National Research Council in the early months of 1945, and clin-

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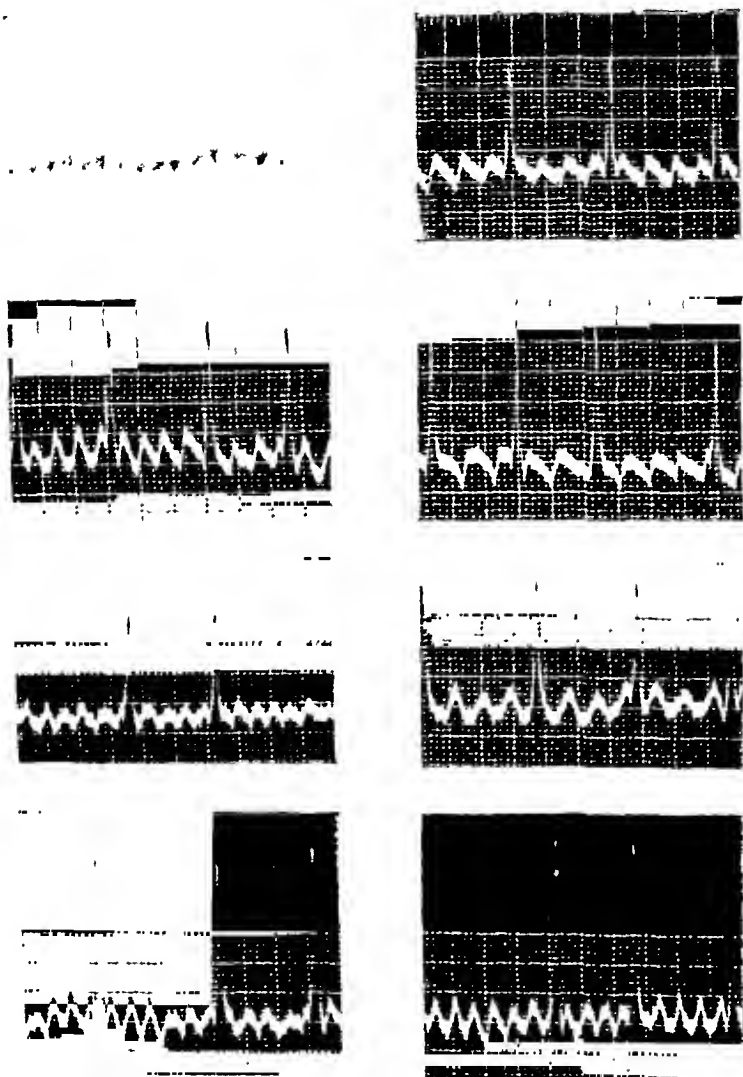
ical tests were carried out under its auspices during the spring, summer, and fall months. The present report constitutes a summary of the results.

**QUININE SULPHATE.** Quinine sulphate was administered to 34 cases of cardiac arrhythmia, mostly auricular fibrillation, to test its efficacy as compared with quinidine sulphate in the prevention and control of the arrhythmia and in reducing the auricular rate. The quinine was apparently effective in only a small minority of the cases (including 4 of 20 patients with auricular fibrillation, 1 of 4 patients with auricular paroxysmal tachycardia, 1 of 4 patients with ventricular paroxysmal tachycardia, and 3 of 6 patients with frequent ventricular premature beats) and was clearly inferior to quinidine.

**PURE (SYNTHETIC) QUINIDINE SULPHATE DIHYDRATE AND SYNTHETIC DIHYDROQUINIDINE SULPHATE DIHYDRATE.** Much more important than the question of quinine has been that of the clinical efficacy of the pure quinidine sulphate and the "impurity" of the dihydroquinidine found to make up 24 per cent of the commercial quinidine in ordinary routine use. A study reported by Lewis and associates (1) twenty-five years ago had already indicated favorable action by both these pure preparations. Recent reports by Scott, Anderson, and Chen (2) and by Woodard, Hagan, and Montgomery (3) concerning the toxicity of these preparations in cats, mice, rats, and dogs showed more effect from the dihydroquinidine than from the quinidine but not sufficient to preclude its clinical trial.

**THE METHOD OF PRESENT CLINICAL STUDY.** This consisted of two parts, the second of which was by far the more important. As in the tests with quinine, one series of nine patients with frequent paroxysms of auricular fibrillation, auricular flutter, or ventricular tachycardia or with frequent premature systoles of ventricular origin (Group A) was given in succession rations of commercial quinidine (0.2 to 0.4 gram), synthetic quinidine (0.2 to 0.4 gram), dihydroquinidine (one half the dose, i.e., 0.1 to 0.2 gram) three or four times a day for a few days at a time, with several days' interval between the courses, in order to test the relative inhibiting effects of these drugs. Both synthetic quinidine and dihydroquinidine proved to be as effective as commercial quinidine in the prophylactic control of cardiac arrhythmias.

A second series of 28 patients with persistent auricular flutter, coarse auricular fibrillation, or ventricular paroxysmal tachycardia (Group B) was given sequentially courses of commercial quinidine, synthetic quinidine, and dihydroquinidine in order to test their effect on the auricular rate (and incidentally on the ventricular rate in auricular fibrillation and flutter) as Lewis and his associates (1) had done. After a control electrocardiogram had been taken, 0.4 gram (6 grains) of synthetic quinidine sulphate was given every two hours for four doses, a follow-up electrocardiogram being taken two hours after each dose just prior to the administration of the next dose (see figure 1). In this way it was possible to plot the auricular rate on a chart, the ordinates of which showed the percentage of slowing of the auricular rate and the drug dosage in grams. The time factor was a constant, as mentioned above. Some of these charts and also composite curves are reproduced in this paper (see figures 2-4).



Before Quinidine

After Quinidine

FIG 1. THE EFFECT OF COMMERCIAL QUINIDINE, SYNTHETIC QUINIDINE, DIHYDROQUINIDINE AND QUININE ON THE AURICULAR RATE OF A GROUP B PATIENT

1st row. Effect of Commercial Quinidine Sulphate on the Auricular Rate. Single Dose: 0.4 Gram. Auricular Rate Before Drug: 510; Auricular Rate 2 hrs. after Drug: 375

2nd row: Effect of Synthetic Quinidine Sulphate on the Auricular Rate. Total Dose: 0.8 Gram. (0.2 Gram every two hrs.) Auricular Rate before Drug: 450; Auricular Rate  $\frac{1}{2}$  hr. after Completion of Course: 330

3rd row: Effect of Dihydroquinidine on the Auricular Rate. Single Dose: 0.4 Gram. Auricular Rate before Drug: 510; Auricular Rate 2 hrs. after Drug: 345

4th row: Effect of Quinine Sulphate on the Auricular Rate. Total Dose: 0.8 Gram. (0.2 Gram every 2 hrs.) Auricular Rate before Drug: 480 Auricular Rate;  $\frac{1}{2}$  hr. after Completion of Course: 510

The same procedure was carried out after an interval of one day with dihydroquinidine, but at only one half the dosage as a rule (0.2 gram, 3 grains), and, finally, commercial quinidine sulphate was administered after another interval of one day in the same dosage as that of the synthetic quinidine (0.4 gram, 6 grains).

In order better to record the auricular waves a special exploring electrode was as a rule used over the region of the upper part of the right auricle just to the right of the sternum, usually in the CR<sub>1</sub> position. Electrocardiograms that failed to show the auricular waves clearly were discarded. Measurements were

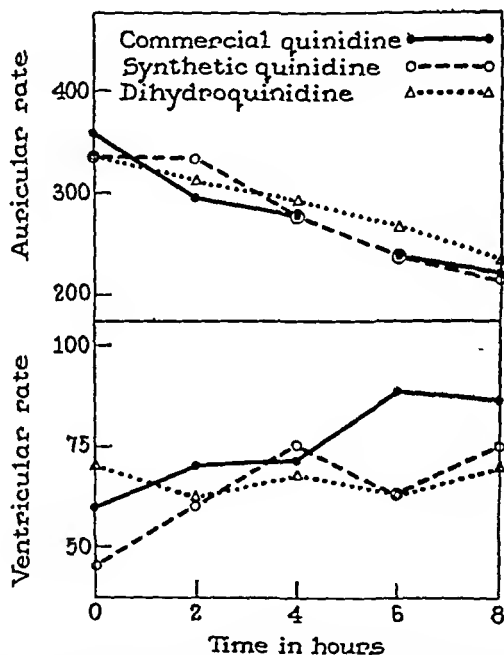


FIG. 2. THE ACTION OF COMMERCIAL QUINIDINE, SYNTHETIC QUINIDINE AND DIHYDROQUINIDINE ON THE AURICULAR AND VENTRICULAR RATES OF A PATIENT WITH AURICULAR FIBRILLATION

repeated by the same observer and independently recorded by another observer, average rates being then determined. Table 1 shows an example of these measurements in one case.

Table 2 summarizes the data in all 28 patients. Figure 1 shows characteristic electrocardiograms before and after the drug administration, and figures 2 to 4 are curves constructed from the charts of measurements illustrated in table 2.

It is evident that both synthetic quinidine and dihydroquinidine (in contrast to quinine) have an effect on the auricular rate in patients with auricular fibrilla-

tion comparable to that produced by commercial (U.S.P.) quinidine, the synthetic quinidine in the same dosage as that of commercial quinidine and the dihydroquinidine in one half the dosage.

An important finding revealed by these clinical tests is well demonstrated by the figures: the curves depicting the speed of slowing of the auricular rate rise quickly with the first two doses and then flatten out. This shows that as the dose increases beyond a certain range the response becomes less sensitive. The in-

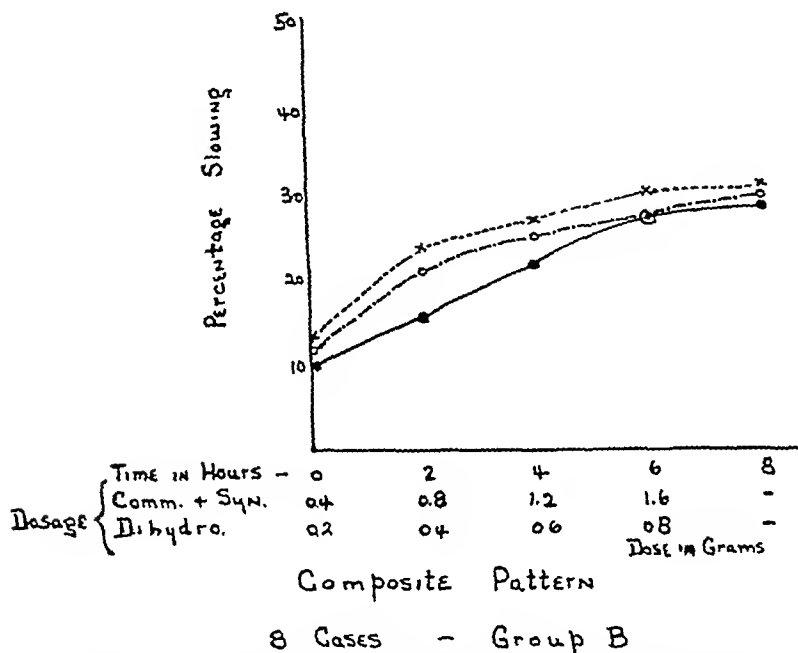


FIG. 3. THE COMPOSITE PATTERN PRODUCED BY THE EFFECT OF COMMERCIAL QUINIDINE, SYNTHETIC QUINIDINE AND DIHYDROQUINIDINE ON THE AURICULAR RATE IN LIGHT PATIENTS OF GROUP B

○-○-○ Commercial, x-x-x Synthetic ●-●-● Dihydroquinidine

frequency of toxic effects in the present clinical study suggests that fairly large doses might be safe as well as more effective, provided close and careful observation is exercised.

Toxic symptoms were looked for but were too inconstant and mild to be of any value in the comparative tests.

COMPARISON OF DURATION OF ACTION OF COMMERCIAL AND SYNTHETIC QUINIDINE. Observations were made on the circus rates in 5 patients with auricular fibrillation. There were 3 cases of coronary heart disease and 2 of rheumatic

mitral stenosis. The same amount of commercial and synthetic quinidine was administered in each instance in a single dose; this varied from 0.4 to 0.8 Gm. The 2 drugs were given on successive days when the dose was 0.4 Gm.; if it was

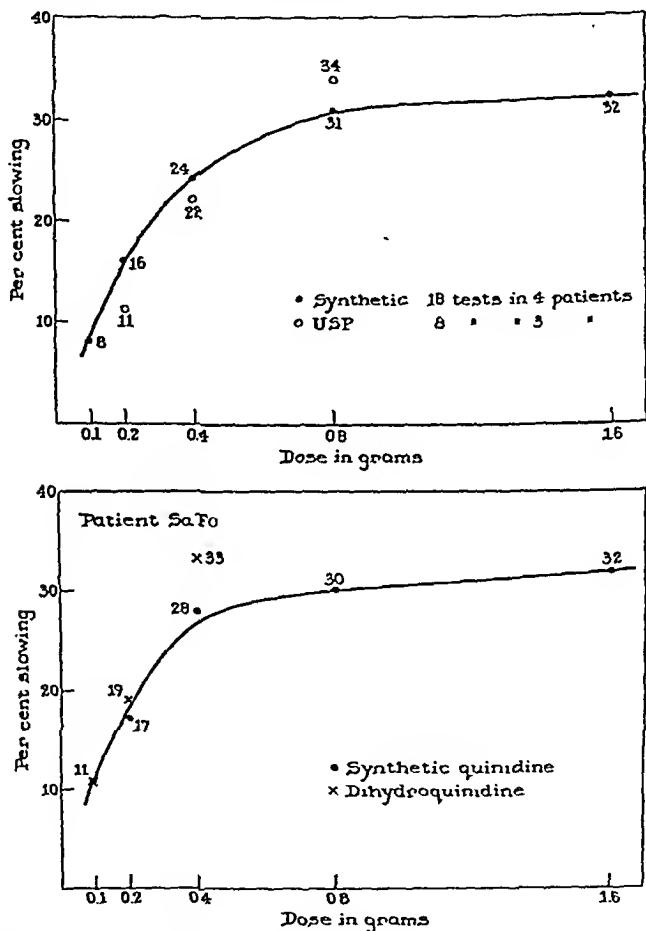


FIG. 4. THE EFFECT OF COMMERCIAL QUINIDINE AND SYNTHETIC QUINIDINE ON THE AURICULAR RATE IN SEVERAL PATIENTS WITH AURICULAR FIBRILLATION  
THE EFFECT OF SYNTHETIC QUINIDINE AND DIHYDROQUINIDINE ON THE AURICULAR RATE IN A PATIENT WITH AURICULAR FIBRILLATION

greater, 2 days were allowed to elapse between their administration to avoid possible cumulative action. When this precaution was taken, the rate of the circus in the control records was quite constant for each patient. After a control

electrocardiogram had been taken, the drug was given at once. Electrocardiograms were made thereafter at intervals of 1 or 2 hours, for 10 to 17 hours. The results are pictured in fig. 5.

In cases 1, 3 and 4, the curves of the action of the 2 drugs are almost superimposed. In cases 2 and 5, the maximal effect of commercial quinidine is slightly delayed; but after 3 to 5 hours, the curves are alike. This occasional slight discrepancy in the intensity of action during the first few hours may reasonably be ascribed to variations in the rate of absorption from the digestive tract.

TABLE 1  
Case B. D.

DRUG		TIME INTERVALS IN HOURS				
		0	2	4	6	8
Commercial quinidine sulphate	Auricular rate	360	333	273	265	226
	Percentage slowed		8	24	26	37
	Ventricular rate	78	80	82	99	87
	Dosage (gm.) (total 1.6)	0.4	0.4	0.4	0.4	
Synthetic quinidine sulphate	Auricular rate	383	311	265	260	220
	Percentage slowed		19	31	32	43
	Ventricular rate	85	86	108	105	83
	Dosage (gm.) (total 1.6)	0.4	0.4	0.4	0.4	
Dihydroquinidine	Auricular rate	403	311	276	275	247
	Percentage slowed		23	32	32	39
	Ventricular rate	65	78	102	83	108
	Dosage (gm.) (total 0.8)	0.2	0.2	0.2	0.2	

It appears, then, that the duration of action of commercial and synthetic quinidine is similar.

#### CONCLUSIONS

1. Synthetic quinidine sulphate and dihydroquinidine can be used in the clinic as safe and effective substitutes for commercial (U.S.P.) quinidine in the treatment of cardiac arrhythmias.

2. Synthetic and commercial quinidine show a similar duration of action.

mitral stenosis. The same amount of commercial and synthetic quinidine was administered in each instance in a single dose; this varied from 0.4 to 0.8 Gm. The 2 drugs were given on successive days when the dose was 0.4 Gm.; if it was

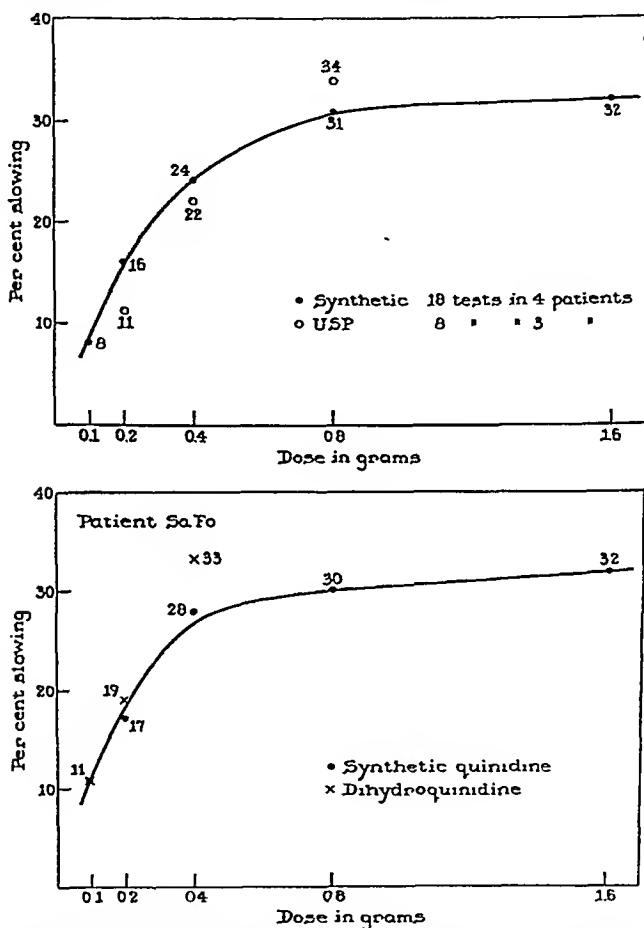


FIG. 4. THE EFFECT OF COMMERCIAL QUINIDINE AND SYNTHETIC QUINIDINE ON THE AURICULAR RATE IN SEVERAL PATIENTS WITH AURICULAR FIBRILLATION  
THE EFFECT OF SYNTHETIC QUINIDINE AND DIHYDROQUINIDINE ON THE AURICULAR RATE IN A PATIENT WITH AURICULAR FIBRILLATION

greater, 2 days were allowed to elapse between their administration to avoid possible cumulative action. When this precaution was taken, the rate of the circus in the control records was quite constant for each patient. After a control







15	New York	Thyroid post-op	16 in 8 hrs	265	220	17		16 in 8 hrs	290	220	34		0.8 in 8 hrs	111	276	33
16	Boston	Arterioscl	16 in 8 hrs	310	220	29		16 in 8 hrs	300	208	30		0.8 in 8 hrs	295	210	30
17	Boston	? Chronic Cor Pulmonale	16 in 8 hrs	310	220	29		16 in 8 hrs	300	208	30		0.8 in 8 hrs	310	220	29
18	Chicago	R II D	16 in 8 hrs	135	185	13		16 in 8 hrs	120	265	37		0.8 in 8 hrs	130	310	28
19	Chicago	Hyper and Cor	0.8	160	235	38	diarrhea	0.8	320	210	25		0.1	325	285	12
20	Cleveland	R II D	0.1	500	370	26		0.8	160	340	26		0.1	500	350	30
21	Cleveland	R II D						0.6	110	345	22		0.6	110	310	23
22	Cleveland	Cor II D						0.4	341	273	20		0.1	349	273	20
23	Cleveland	R II D											0.4	428	306	28
24	Cleveland	Hyper II D											0.1	517	360	30
25	New York	Arterioscl	0.1 in 6-8 days	377	287	24		16 in 15-20 days	500	286	43					
26	New York	R II D	0.8 in 12 days	101	278	31		0.8 in 12-16 days	129	264	38					
27	New York	R II D	0.8 in 9-12 days	517	401	22		0.8 in 12-16 days	536	417	22					
28	New York	Arterioscl						0.8 in 12-16 days	319	235	26					

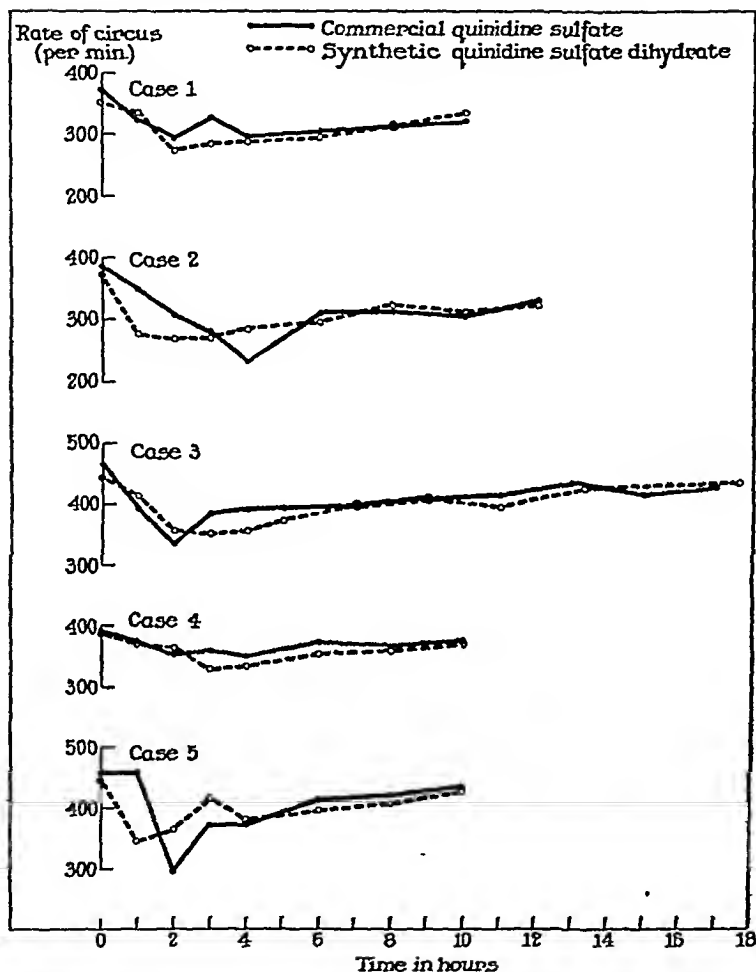


FIG. 5

Case 1. Rheumatic heart disease, mitral stenosis and auricular fibrillation. Initially fully digitalized and continued on maintenance dosage. Patient received 0.6 Gram quinidine sulfate, then 0.6 Gram quinidine sulfate dihydrate 2 days later.

Case 2. Coronary heart disease and auricular fibrillation. Initially fully digitalized and continued on maintenance dosage. Patient received 0.6 Gram quinidine sulfate, then 0.6 Gram quinidine sulfate dihydrate 2 days later.

Case 3. Coronary heart disease and auricular fibrillation, not digitalized. Patient received 0.8 Gram quinidine sulfate, then 0.8 Gram quinidine sulfate dihydrate 2 years later.

Case 4. Coronary heart disease and auricular fibrillation. Initial fully digitalized and continued on maintenance dosage. Patient received 0.4 Gram quinidine sulfate dihydrate, then 0.4 Gram quinidine sulfate next day.

Case 5. Rheumatic heart disease, mitral stenosis and auricular fibrillation. Initially fully digitalized and continued on maintenance dosage. Patient received 0.4 Gram quinidine sulfate dihydrate, then 0.4 Gram quinidine sulfate next day.

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# STUDIES ON THE EFFECT OF REPEATED ADMINISTRATION OF 1-AMINO-1-PHTHALIDYLPROPANE HYDROCHLORIDE AND OF THE EFFECTS OF THE DRUG UPON ARTERIAL BLOOD PRESSURE AND RESPIRATION<sup>1</sup>.

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The chemical 1-amino-1-phthalidylpropane hydrochloride was first synthesized by Ulyot and his associates (1). They obtained two racemates which they designated by the letters A and B. The acute toxicity of these racemates as well as their actions in raising the pain threshold have been investigated in rats and cats by Fellows (2) and Fellows and Cunningham (3). In experimental animals, the circulatory, gastro-intestinal, cardiac, and respiratory actions of "phthalidyl"<sup>2</sup> were found to be negligible (4). Because it elevated the threshold pain stimulus more than did aminopyrine and because it had a lower acute toxicity than had aminopyrine, Fellows recommended that 1-amino-1-phthalidylpropane hydrochloride be given a clinical trial as an analgesic agent.

Due to the fact that no chronic toxicity studies have been made and only a limited number of experiments have been done on the pharmacology of these racemates, including a comparison of their actions with those of other analgetics, this investigation was undertaken.

**METHODS.** In the chronic toxicity studies fourteen normal female dogs weighing between 5 and 16 kgm. were used. After the animals were de-wormed and immunized against distemper, they were studied from two to four weeks before the drugs were administered. One group of animals was given 1-amino-1-phthalidylpropane hydrochloride (racemate B) either orally or intravenously daily (except Sundays) and another group was given racemate A in the same way (see table 1).

Phenolsulphonphthalein and bromsulphalein excretions, hemoglobin estimations, hematocrit determinations, erythrocyte and leukocyte counts were made one or more times before the drug was given and thereafter every 10 to 14 days throughout the experiment. Differential leukocyte counts as well as occasional reticulocyte counts were made at the same time. At the end of the experiment the animals were sacrificed and gross as well as microscopic examinations of most organs were made.

Sixteen cats and sixteen dogs were used in the pharmacological studies of the acute effects of these drugs. Four of the cats were given continuous ether anesthesia by inhalation, three were given urethane by stomach tube, nine were decerebrated while under ether anesthesia after which the anesthetic was discontinued and one was given procaine hydrochloride as a local anesthetic. Five of the dogs were anesthetized with ether by inhalation and eleven

<sup>1</sup> This research was made possible through a grant from Smith, Kline and French Laboratories.

<sup>2</sup> Phthalidyl is the name coined by Dr. Fellows for 1-amino-1-phthalidylpropane hydrochloride.

with sodium pentobarbital intravenously. Except in the one instance in which local anesthesia was used, tracheal cannulae were inserted into the tracheas of all of the animals for the following reasons: because of the convenience in continuing the inhalation anesthesia; furthering free respiration and in carrying out artificial respiration when it was needed. The blood pressure was recorded with a mercury manometer attached by means of heavy rubber tubing to a cannula placed in the left carotid artery. In the one experiment in which procaine was employed the femoral artery was used. Sodium citrate was employed in the system as the anticoagulant. In cats the respirations were recorded by fastening a ligature to the diaphragm, the ligature passing under a pulley to a muscle lever. In dogs a pneumograph placed about the chest wall was connected by means of rubber tubing, to a large recording tambour. Records of the splenic volume were also taken in most experiments on the dogs by means of a spleen oncometer connected to a modified Brodie bellows. A chrono-

TABLE I

DOG	WEIGHT IN KG		ROUTE OF ADMINISTRATION OF DRUG	MG PER KG	NUMBER OF DAYS	AVERAGE HEMOGLOBIN IN GM/100 CC OF BLOOD		AVERAGE HEMATOCRIT VOLUME PER CENT R B C		ERYTHROCYTES IN THOUSANDS/MM <sup>3</sup>		LEUCOCYTES IN THOUSANDS/MM <sup>3</sup>		NUCLEATED R B C /1000 W.B.C	
	Be-fore	End				Con-trol	Ex-peri-men-tal	Con-trol	Ex-peri-men-tal	Con-trol	Ex-peri-men-tal	Con-trol	Ex-peri-men-tal	Be-fore	Dur-ing
1B	15	16.0	Oral	50*	49*	17.0	17.4	76	69	6,737	7,012	12.0	10.2	0	0
3B	16	16.0	Oral	50*	49*	16.2	17.1	79	77.6	6,925	7,555	11.8	9.2	0	30
4B	10	12.5	Oral	100	85	16.2	16.7	68	69	6,600	7,887	9.3	12.8	0	+
5B	10	12.0	Oral	100	85	15.2	15.9	80	67	6,478	6,753	10.7	11.8	0	+
7B	6	7.5	Oral	50†	55†	15.3	13.9	68	64	6,500	6,331	12.6	9.5	+	+
9B	9	9.0	Intravenous	50	74	18.0	17.6	81	79	7,115	7,764	12.1	12.0	26	62
10B	8.5	10.5	Intravenous	50	74	18.7	17.7	72	73	8,800	7,410	11.2	11.7	0	+
1A	9	14.0	Intravenous	50	61	13.5	16.4	57	53.5	5,581	6,545	12.7	8.2	0	0
3A	5	9.5	Oral	100‡	29‡	16.3	17.3	70	68	7,500	7,273	14.8	10.2	+	6
4A	8.5	9.5	Oral	100	61	14.0	17.1	58	60	6,400	6,551	16.8	10.6	+	6
5A	8	10.5	Oral	100	61	15.0	16.4	64	50	6,020	6,145	11.6	13.4	+	190
6A	9	13.0	Oral	100	61	13.6	16.4	55	56	6,125	6,555	14.5	22.9	+	12
7A	10	13.0	Intravenous	50	61	14.8	16.2	60	52	6,557	6,870	19.3	13.8	0	0
8A	11	16.0	Intravenous	50	61	13.4	16.0	54	65	5,550	7,155	10.4	11.6	+	+

\*Animal received 100 mgm/kgm for an additional 23 days

†100 mgm/kgm an additional 24 days.

‡50 mgm/kgm intravenously, 32 days.

+Nucleated R B C found

graphic marking key, indicating intervals of either 6 or 10 seconds was placed at the zero blood pressure level. The drugs were injected into the femoral vein.

Since Fellows (4) found aminopyrine more toxic than 1-amino-1-phthalidylpropane hydrochloride and because of its solubility, we also selected this analgetic for studies comparing its pharmacological actions with those of the two racemates.

**RESULTS. Chronic Toxicity.** None of the dogs given 1-amino-1-phthalidylpropane orally showed any acute toxic effects. All of the animals in which the administrations were made intravenously, however, responded by excessive salivation with some frothing, marked dilation of the pupils, slowed respiration, noticeable muscular tremors, and muscular rigidity. Upon repeated injections all these effects became less noticeable. (In one case mild convulsions occurred from racemate B.)

By means of a stop-watch the time interval between the beginning of the injection of the drug and excess salivary secretion was determined 10 times in each of three dogs, these averaging 8.7, 9.7, and 12.2 seconds. We believe the increased secretion of saliva is due to the bitterness of the drug and its onset is an indication that the drug is being excreted by the salivary glands.

Usually when animals 7A and 8A were given intravenous injections they defecated and animal 8A always urinated as well.

As far as we were able to determine, neither oral nor intravenous administrations either of racemate A or of B of 1-amino-1-phthalidylpropane hydrochloride taken over a period of months produce harmful effects. The animals showed no loss in weight, or appetite and they remained alert and exceedingly active (table 1).

Throughout the series of experiments the phenolsulphonphthalein and bromsulphalein excretions, the blood hemoglobin and the hematocrit readings remained within the normal range. In all instances the number of erythrocytes, reticulocytes, and leukocytes per cu. mm. of blood remained within the limits of variation found by Deinard and her associates, (5) in normal dogs.

No mention is made by Deinard and her associates (5) as to whether or not nucleated red blood cells were found in the blood smears of their normal animals. In the smears made from our fourteen animals, seven showed the presence of nucleated red blood cells during the preliminary weeks of study and eleven after the chemical had been given for some time (see figure 1). In a few of the animals these nucleated cells appeared to become more numerous after the drug had been administered yet this was not a constant result. In the eleven animals showing nucleated red blood cells, the number of nucleated red blood cells varied on these examinations from one cell per smear to 190 cells per 1000 white blood cells (see table 1). In as much as these cells were normally present in the blood stream of some of these animals before they were given the drug we are inclined to believe that the chemical was not responsible for any increases occurring after administration of the drug had begun. Autopsy and microscopic examinations of the lungs, heart, spleen, urinary bladder, liver, pancreas, stomach, intestine, thyroid glands, kidneys and adrenal glands revealed nothing abnormal which could be attributed directly to the chemical.

**RESPIRATION.** Both racemates A and B of 1-amino-1-phthalidylpropane hydrochloride depress respiration in normal as well as in anesthetized experimental animals but this depressant action is much more noticeable in animals under ether, urethane or pentobarbital anesthesia than in either unanesthetized or decerebrated ones. In cats under ether or urethane anesthesia and in dogs under ether or pentobarbital anesthesia the first injection of 50 mgm./kgm. of racemate B or of racemate A usually caused some slowing of respiration and a second or third injection usually caused complete stoppage of respiration in expiration (figures 2 and 3). Some of our decerebrated cats tolerated as many as six injections of 25 mgm./kgm. and two of 50 mgm./kgm. of the drug without its producing marked slowing. The record in figure 2, is presented as being

typical of the majority of the experiments. In this figure the first injection of racemate A caused a cessation of respiration in expiration which lasted for about 10 seconds. Upon resumption the respiration continued to be slow. In a few instances a slight reflex acceleration of respiration occurred during the fall in blood pressure after which the respirations were less frequent than in the control.

Aminopyrine, unlike 1-amino-1-phthalidylpropane hydrochloride, when injected intravenously in doses of 50 mgm./kgm., caused a decided increase in the rate of respiration whether the animal was anesthetized or decerebrated. As an

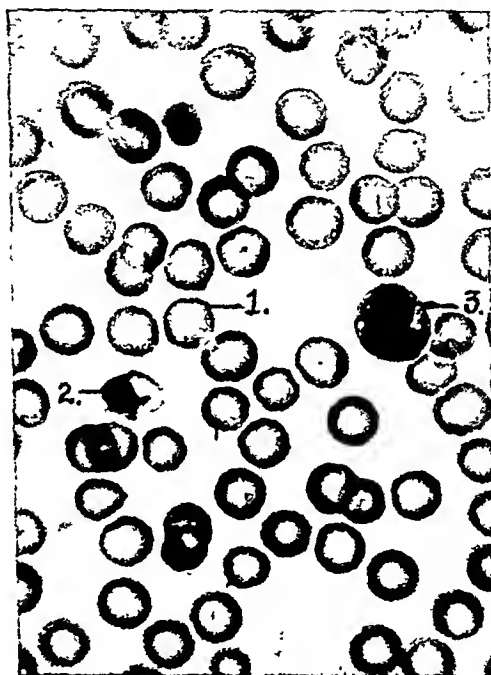


FIG. 1. A PHOTOMICROGRAPH OF THE BLOOD OF DOG 5A. MAGNIFICATION 900 X  
1. Normal red blood cell. 2. Nucleated red blood cell. 3. Large lymphocyte.

example, in one decerebrated cat the respirations were decreased from 57 to 27 with 50 mgm./kgm. of racemate A but a similar dose of aminopyrine increased the respirations to 141 per minute. In figure 2, at 2, 50 mgm./kgm. of aminopyrine was injected intravenously after the respirations had been slowed by racemate A as seen at 1 in this figure, and as a result the respirations increased from 9 to 48 respirations per minute continuing at this rate until another injection of racemate A was made.

**ARTERIAL BLOOD PRESSURE.** Both racemate A and B of 1-amino-1-phthalidylpropane hydrochloride temporarily lower the arterial blood pressure in experi-



mental animals (see figures 2 and 3). In the sixteen cats, thirty-two injections of racemate A and twenty-six injections of racemate B were made resulting in average percentage decreases in blood pressure for doses of 10, 25, 50 and 100

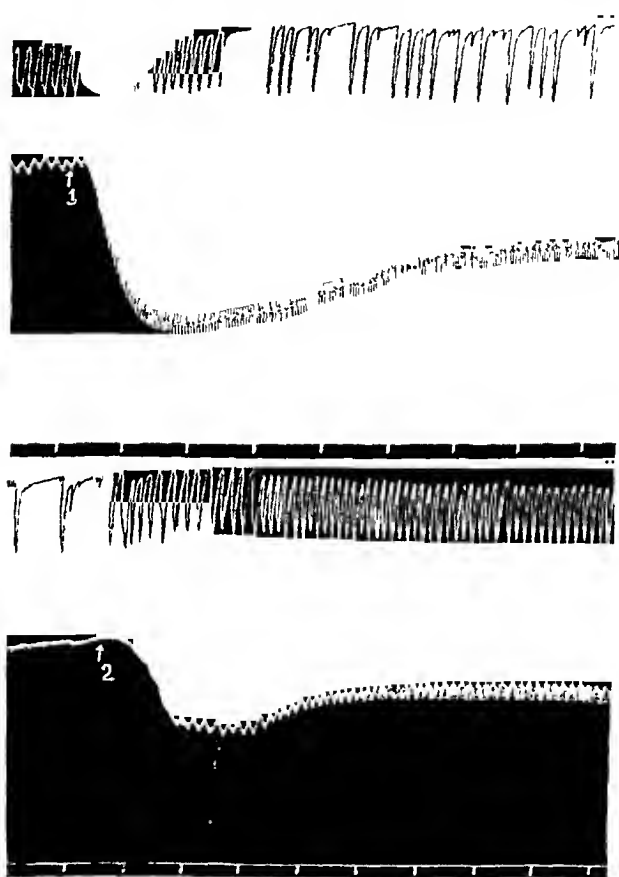


FIG. 2. CAT, URETHANE ANESTHESIA

The top curves in each record are the respirations, the middle curves, the blood pressures with a mercury manometer, and the bottom lines zero blood pressure and the time intervals of 10 seconds. In the respiratory records the up stroke indicates inspiration. In the upper record at 1, 50 mgm./kgm. of racemate A were injected intravenously and in the lower record at 2, 50 mgm./kgm. of aminopyrine were injected intravenously.

mgm./kgm., for B of 18, 31, 35, and 50 respectively, and for A, 27, 44, 57 and 60 respectively. Twenty-eight injections of racemate A and eleven injections of racemate B were made in twelve dogs. The average percentile falls in blood

pressure following the injections of 25, 50, and 100 mgm./kgm. of racemate A were 32, 43 and 62 respectively and with racemate B in doses of 50 and 100 mgm./kgm., they were 33 and 56 respectively.

Usually the fall in blood pressure was brief and within a few minutes it was either back to the control level or above it. In the experiments on dogs with the use of racemate A, 27 of the 28 injections produced a sudden fall in blood pressure followed by a prolonged rise. With the use of 25 mgm./kgm., this rise averaged



FIG 3 DOG, 21 KGm. ETHER ANESTHESIA

Top record is that of respiration in which the down stroke is inspiration. Middle record is that of blood pressure with a mercury manometer, and the bottom line is that of zero blood pressure and the time in intervals of 6 seconds. At A, 50 mgm./kgm. of racemate A were injected intravenously. Electrocardiograms were taken at E.

20 per cent but when 50 mgm./kgm. were injected the average rise was 44 per cent (see figure 3). The rise in blood pressure in figure 3 may have been partly due to anoxia, however, this cannot be the only cause since the larger rises in blood pressures were most frequently seen in the animals under continuous artificial respiration. In most instances during this rise in blood pressure there were acceleration of the heart and a decrease in the volume of the spleen.

The average decreases in blood pressure following the intravenous administra-

tion of 25, and 50 mgm./kgm. of aminopyrine were 20 and 27 per cent respectively. In dogs 50 mgm./kgm. of aminopyrine caused an average prolonged decrease in blood pressure of 24 per cent. Generally speaking the arterial blood pressure after aminopyrine gradually returned to the normal control level but rarely did it exceed it.

Racemate A was found to be approximately twice as active upon the respiratory and cardiovascular systems as was racemate B, as can be seen in the percentile falls in blood pressure with similar dosages. In figure 4, a decerebrated cat was used and at 1, 10 mgm./kgm., of racemate B, was injected, and, at 2, a similar amount of racemate A was administered. In 1 the arterial blood

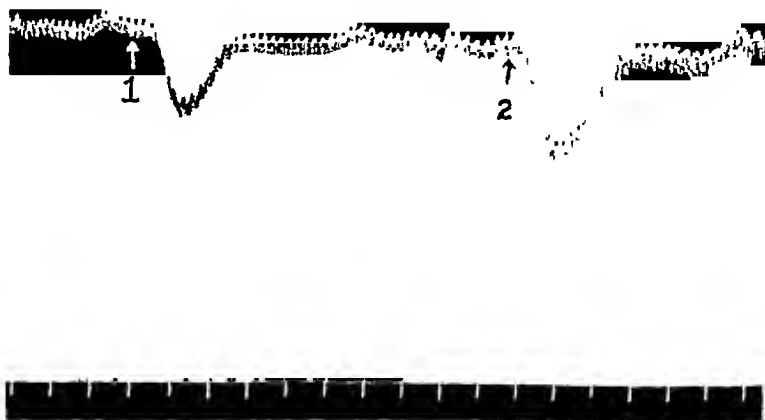


FIG. 4. CAT 3.6 KGm. DECEREBRATED UNDER ETHER ANESTHESIA

The top curve is that of the blood pressure taken with a mercury manometer. The bottom line indicates zero blood pressure and the time in intervals of 10 seconds. At 1, 10 mgm./kgm. of racemate B were injected intravenously and at 2, 10 mgm./kgm. of racemate A were similarly injected.

pressure decreased only 24 per cent where as in 2 the decrease amounted to 33 per cent.

Five experiments were performed in which changes in ventricular activity were recorded by means of a Cushny myocardiograph. During the entire experiment the dogs were kept under pentobarbital anesthesia and artificial respiration. In these animals 2 injections of racemate B, 11 injections of racemate A and 7 injections of aminopyrine were made, the dosage in each case being 50 mgm./kgm. The intravenous injection of either racemate A or B of 1-amino-1-phthalidylpropane hydrochloride caused a prompt fall in blood pressure with a simultaneous slowing in cardiac rate and decreases in both the general tonus and the force of the contractions of the ventricles. As the blood pressure increased above the control level, following the fall, the force of the contractions of

the heart also increased. This increase in heart action may have been due to the change in blood pressure. These changes can best be seen in figure 5. At 1, 50 mgm./kgm. of racemate A were injected intravenously in a 10 kgm. dog. The blood pressure fell abruptly from 126 to 44 mm. Hg and then increased to 219 mm. Hg as seen in 2. As the blood pressure decreased the heart rate decreased from 36 to 19 contractions per 30 seconds. In 11 decerebrated and urethanized cats racemate A was injected 28 times and B 8 times. Racemate B in doses of 25, 50 and 100 mgm./kgm. caused an average decrease in the heart rate of 8, 15 and 40 percent respectively. Racemate A injected in 25 and 50 mgm./kgm. doses caused an average decrease in heart rate of 24 and 35 per cent respectively.

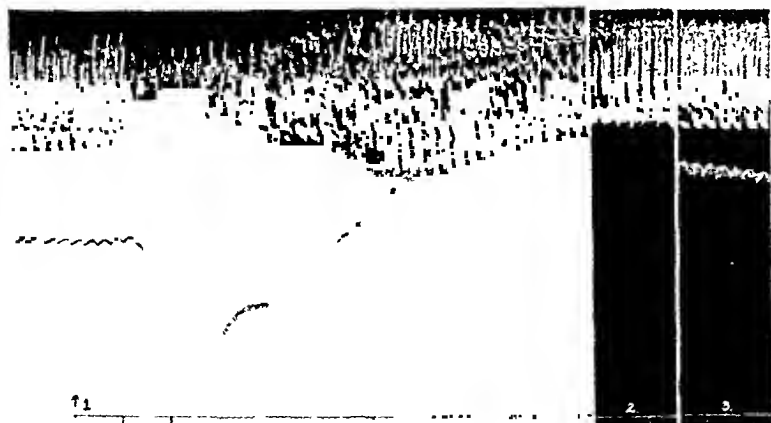


FIG. 5. DOG 10 KGm. PENTOBARBITAL SODIUM ANESTHESIA AND ARTIFICIAL RESPIRATION

Top line is record of the ventricle taken with a Cushny myocardiograph, middle curve that of blood pressure taken with a mercury manometer and the bottom line is the time in intervals of 10 seconds and zero blood pressure. At 1, 50 mgm./kgm. of racemate A were injected intravenously. Curve 2 was taken 30 seconds after the end of 1 and 3, 90 seconds after 2.

Aminopyrine was injected in doses of 50 mgm./kgm. 11 times in eleven cats. In these the average increase in heart rate was 21 per cent. In the five dogs in which the heart was studied with the use of a Cushny myocardiograph 7 injections of 50 mgm./kgm. of aminopyrine were made causing acceleration of the heart in each instance and an increase in the cardiac tone. Electrocardiographic studies done on dogs, using lead II, revealed nothing unusual. The changes in heart rate as given above were noted. No demonstrable change in the P-R interval in the electrocardiogram was seen. The T-waves appeared definitely higher than normal during the period of excessive slowing of the heart.

The exact point of action of 1-amino-1-phthalidylpropane hydrochloride in producing the fall in blood pressure was not determined. The volume changes of

the spleen following the intravenous injections of the drug gave very little information, because of their variability. In some instances the spleen decreased during the fall in blood pressure (probably a passive effect) in others the spleen increased in volume. During the increase in blood pressure after the temporary fall the spleen always decreased in size thus taking part in the general vasoconstriction. The fall in blood pressure may be due partly to the action of the drug on the heart muscle since some slowing of the heart was usually seen. This slowing is not a vagal effect since neither cutting the vagus nerves nor the administration of atropine in doses adequate to paralyze the vagus nerves had any influence on this response. Following the injection of aminopyrine the spleen usually increased in volume during the fall in blood pressure and gradually returned to its control size with the return of blood pressure to the control level.

#### SUMMARY AND CONCLUSIONS

(1) Neither racemate A nor racemate B of l-amino-l-phthalidylpropane hydrochloride, when administered daily for several months, produces changes in the tissues of otherwise normal dogs.

(2) Nucleated red blood cells are probably found normally in many dogs' blood. The ratio of these cells may be as high as 1 to 50 W.B.C. and in one of our treated dogs it rose to 1.9 to 10 W.B.C.

(3) Small doses of the above racemates slow respiration in normal, decerebrated and anesthetized animals and large doses cause death by respiratory paralysis. Comparative doses of aminopyrine, similarly administered, accelerate respiration.

(4) All doses of racemates A and B of l-amino-l-phthalidylpropane hydrochloride decrease arterial blood pressure. A subsequent increase in arterial blood pressure usually occurs. Aminopyrine given in comparative doses lowers the blood pressure but to a lesser degree and subsequent increases in blood pressure above that of the control are rarely seen.

(5) Racemates A and B slow the heart by an action which is not abolished by either atropine or vagotomy. The T-wave in the electrocardiogram may be increased in height.

(6) The changes produced in the spleen by these drugs are variable and inconclusive. The splenic volume increased during the fall in blood pressure following intravenous injection of aminopyrine.

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# A COMPARISON OF CERTAIN DIGITALIS GLYCOSIDES

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In view of the increasing clinical use of pure digitalis glycosides and the claims made for the various substances now available it becomes desirable to clarify as far as possible important differences in individual glycosides. To date there is little agreement in reported studies, owing to differences in methods used and criteria for comparison. Visscher and LaDue (1) pointed out that when the basis for comparison was the single intravenous dose required for "full digitalization" in auricular fibrillation there was a remarkably small difference in the molar quantity of ouabain, digitoxin, digoxin and lanatoside C required to produce comparable effects. They added that it was by no means certain that such a comparison would hold for other criteria. Because of the dual mechanism of digitalis action on the heart itself, vagal and direct muscle actions, and subsequent changes in the circulation, evaluation of these drugs in the intact animal becomes difficult.

Cattell and Gold (2) studied the effect of various glycosides on the isolated papillary muscle of the cat, using as a criterion of therapeutic effect increased systolic tension, and of toxicity, subsequent decline which occurred with stronger concentrations. Ouabain and digitoxin were found to be of equal potency, while the activity of the lanatoside compounds was about one tenth as great. However, recently White and Salter (3) using the same technique reported ouabain to have approximately twice the potency of digitoxin. DeGraff and his collaborators (4) have considered the embryonic chick heart a suitable test preparation for cardiac glycosides and have made extensive comparative observations. The appearance of atrioventricular block was the criterion used. By their method they found the concentration ratios for digitoxin, digoxin and lanatoside C to be 1:3:4.5.

The most constant action of digitalis in the intact animal or on heart muscle strips is to shorten the interval between depolarization and repolarization of the muscle membrane with each contraction, and this is evidenced by shortening of the refractory period or of the Q-T interval of the electrogram (5). Such action by ouabain has been studied in man by Apter, Ashman and Hull (6), who found reduction in the gradient magnitude of repolarization ranging from 22 to 48 per cent and this was not always indicated by T wave changes. A study of this phase of digitalis action on strips of turtle ventricle has been reported from this laboratory (5). Using the same technique comparative observations have been made with ouabain, digitoxin, digoxin and lanatoside C.<sup>1</sup> It is stressed that in

<sup>1</sup> Digitoxin was contributed by the Varick Pharmacal Company and by Wyeth, Inc., and lanatoside C (Cedilamid) by the Sandoz Chemical Works, Inc.

these experiments the Q-T interval was recorded from a localized region, the electrode on the muscle being paired with an indifferent electrode placed at a distance of at least 2 cm., and fibre conduction time is not a part of the interval. The degree of shortening found was subject to considerable variation, but the underlying factors have not been ascertained. Intensity of action could not be related to the time the strip had been out of the animal. Nor was there a constant relation of effect to the initial Q-T interval. While long initial intervals are theoretically capable of the greatest shortening actually the greatest percentage reduction observed occurred in strips whose initial intervals were exceptionally short. This variability of response appeared much the same for each of the glycosides studied.

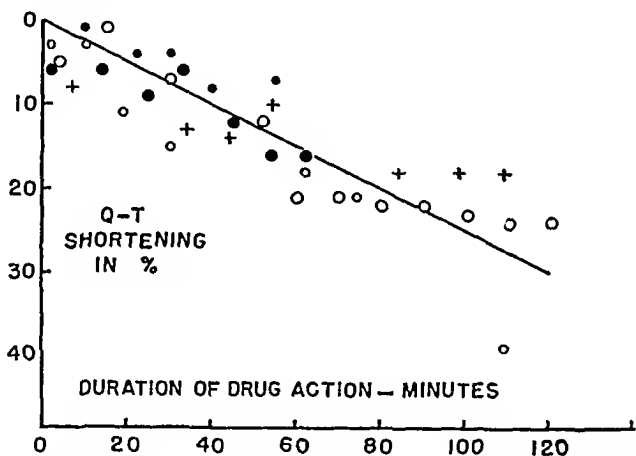
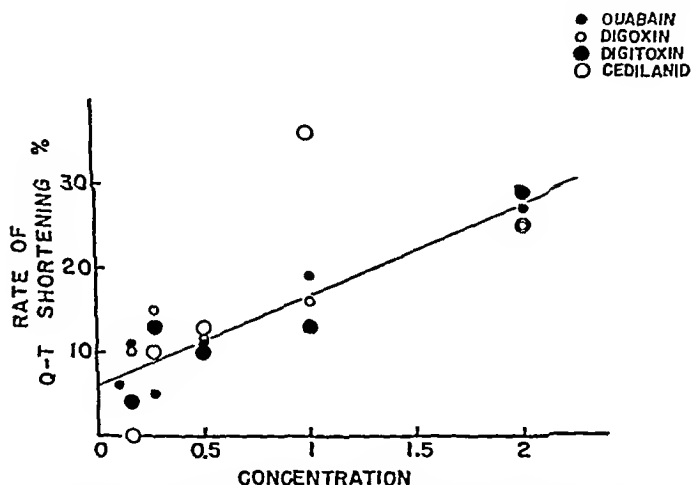


FIG. 1. Shortening of Q-T interval as per cent of initial length plotted against the time after administration of digoxin 0.33 parts per million. The different symbols represent 5 separate experiments. As is typical for all the drugs studied the shortening is approximately proportional to the time for an hour or more. The slope of the line drawn is intended to measure the average rate which is 15% per hour in this case.

The effect of each drug was observed in a series of concentrations ranging from 0.2 to 2.5 parts per million. Usually 4 or 5 strips were tested with each concentration. The strip was rhythmically stimulated for an hour before the drug was added and then observed from 2 to 3 hours. Results are expressed as shortening of Q-T in percent of initial length for the various times. The results for a single series in which digoxin 0.33 parts per million was used is illustrated in figure 1. This a fairly representative group, although in some a single experiment might show greater deviation from the average than is seen in this one. The average shortening per hour for each series was then estimated and these averages plotted against concentration in parts per million, figure 2. With strong concentrations complexes often, but not constantly, ceased to be measurable within an hour, thus terminating the experiment. The line drawn in figure

2 indicates that the rate of shortening is approximately linear with drug concentration but not proportional to it since the line does not pass through the origin. The probable reason for this is that the shortening includes two components, one spontaneous, and one due to drug action. While the period of greatest spontaneous shortening is the first hour of rhythmic stimulation, control experiments have shown that without the drug such shortening usually continues; in three experiments in this series there was an average spontaneous shortening of 8 per cent during the second hour. If such spontaneous shortening still occurs while the drug is acting each datum of figure 2 will include about 8 per cent of spontaneous shortening as well as that due to the drug. In other words, the line will be 8 per cent too high throughout and will have an intercept of approximately





Studies on the influence of these glycosides on contractility that were carried on simultaneously will be considered later. It may be said here that no such uniformity of action, as has been described for the effect on the muscle recovery process, was seen. Ouabain was found to be the most potent, its activity being at least 2.5 times that of digitoxin, giving results in agreement with those of White and Salter rather than those of Cattell and Gold.

#### SUMMARY

The most constant action of digitalis glycosides is to shorten the local Q-T interval. A comparison of digitoxin, ouabain, digoxin and lanatoside C using as a criterion their ability to shorten the Q-T interval of the electrogram indicated that these glycosides were qualitatively and quantitatively similar, and that the magnitude of the effect was proportional to the concentration of the drug.

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# THE ACTION OF N,N-DIBENZYL-BETA-CHLOROETHYLAMINE (DIBENAMINE) IN HYPERTENSIVE DOGS

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The development of a sympatholytic agent having a prolonged action and devoid of undesirable side effects would be of considerable value in the study of various states in which the sympathetic nervous system is implicated. A number of compounds have been previously suggested but these have a short duration of effective action. We previously used the dioxane derivative, F933, which was known to be a sympatholytic agent, in the study of experimental hypertension (1). This drug was found to cause a fall in blood pressure in normotensive and hypertensive dogs and to inhibit the pressor action of administered epinephrine. However, its action was fleeting and it was apparently disturbing to the animal.

Recent investigations have disclosed a number of extremely active pharmacodynamic agents. One of these N,N-dibenzyl-beta-chloroethylamine (dibenamine) has been reported to have a prolonged sympatholytic action with relatively low toxicity and with few alarming side actions. Nickerson and Goodman (2) have reported that dibenamine inhibits the excitatory sympathetic or epinephrine induced reactions. The inhibitory responses are not affected by this drug. They further found that dibenamine prevented cardiac irregularities induced by epinephrine-cyclopropane in dogs (2, 5). A single dose of dibenamine was reported to be capable of suppressing the excitatory effects of epinephrine for as long as 4 days. These favorable properties suggested that dibenamine might be useful in the study of experimental hypertension. We undertook to investigate its effects on the blood pressure in dogs with experimental hypertension of renal origin and in normotensive dogs. The effects of dibenamine on the blood pressure and on the cardiac response to epinephrine were also observed.

**METHODS.** Four dogs with chronic renal hypertension of at least 4 years' duration, two with recent hypertension of less than 4 months' duration and three normotensive animals were used in this study. Blood pressures in these unanesthetized animals were recorded with the Hamilton manometer (3). Electrocardiograms (lead 2) were recorded with a string galvanometer. Dibenamine hydrochloride<sup>1</sup>, 20-40 mgm. per kilo was given slowly intravenously. In studies on the effects of single doses of the drug, epinephrine hydrochloride, 1 mgm., was given intravenously each day for 4 or 5 days. Observations were made on a) the immediate and delayed effect of dibenamine on the blood pressure level of the animal, b) the action of dibenamine on the blood pressure response to epinephrine and c) on the epinephrine-induced ventricular tachycardia.

<sup>1</sup> The dibenamine used in these experiments was generously supplied by Dr. L. S. Goodman of the University of Utah and by Givaudan-Delawanna, Inc., New York City. In the early experiments the drug was dissolved in propylene glycol and in later ones in alcohol. The effects with both solvents were similar.

**RESULTS.** *Effect of a single dose of dibenamine on blood pressure and heart rate.* We observed no significant difference in the response of hypertensive and normotensive dogs to the administration of dibenamine. Injection of dibenamine in 4 animals resulted in a marked and sustained drop in blood pressure lasting for a number of hours and accompanied by varying degrees of tachycardia (fig. 3 and table 1); in one of this group only a fleeting blood pressure fall was seen when 20 mgm. of dibenamine was used instead of 30 mgm. In 2 animals no reduction in blood pressure was seen (fig. 2) and in 1 the reduction was slight and fleeting (e.f.).

TABLE 1

*Response of pulse rate and blood pressure to a single dose of Dibenamine*

DOG	DOSE OF DIBEN- AMINE	INJE- CTION PERIOD	AVERAGE PRE-EXPER- IMENT BLOOD PRESSURE		CONTROL READINGS		LOWEST BLOOD PRESSURE READINGS† AFTER DIBENAMINE			TIME ELAPSED BEFORE MAXIMUM BLOOD PRESSURE FALL*	TIME ELAPSED BEFORE REESTAB- LIZATION AT CONTROL LEVEL	
					Blood pressure	Pulse rate	Blood pressure	Asso- ciated pulse rate				
	mm./ kilo	min.	mm. Hg.		mm. Hg.		beats/ min.	mm. Hg.		beats/ min.		
			S	D	S	D		S	D			
Z-6	20	3	210	120	200	100	108	65	40	260	64 min.	46 hrs.†
Z-38	30	38	200	120	210	120	75	90	45	156	135 min.	48 hrs.†
X-56	20	6	200	110	215	115	100	140	100A	140	89 min.	101 min.§
Z-18	20	4	240	145	240	140	144	140	76	196	550 min.	71 min.§
	30	77			250	158	200	95	30	340	10 min.	28 hrs.†
Z-20	40	36	200	115	225	120	70	200	112B	160		no fall
Z-23	20	2	145	80	150	95	60	75	40	144	84 min.	26 hrs.
Z-31D	20	4	205	105	205	110	85	165	100C	168	400 min.	no fall

S = systolic.

D = diastolic.

\* = from end of injection.

† = based on diastolic pressure.

‡ = wide fluctuations of blood pressure present.

§ = only a transitory fall in pressure.

|| = profound fall in pressure.

A = single reading below 100 mm. Hg was observed (110/55). This lasted 1 minute.

B = single reading of 170/95 lasting 1 minute was noted.

C = single reading of 145/75 lasting 1 minute was noted.

D = partially trained animal.

fig. 1). In addition it was obvious that in some of the animals dibenamine led to an unstable blood pressure with a tendency to wide fluctuations (fig. 4). In one animal in which the greatest depressor effect on blood pressure was seen, we were concerned for several hours that the animal might develop irreversible shock as a result of the prolonged pressure drop. All but one of the animals exhibited general lassitude, inactivity, and definite muscular weakness, which was most pronounced in the hind legs. These signs persisted for one or two days. In several animals in which the injection was made relatively rapidly a period of excitement and irritability immediately preceded the depressor effect. In one,

clonic convulsions, vomiting, urinary and fecal incontinence, as well as stertorous respiration developed. However, in another in which a larger dose (40 mgm. per

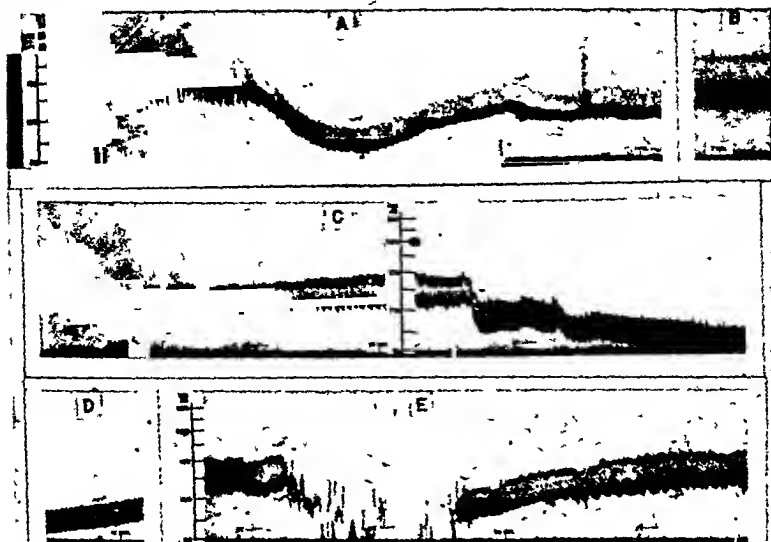


FIG. 1. Segments of a blood pressure record obtained in an unanesthetized trained dog with hypertension of recent origin following the intravenous administration of dibenamine 20 mgm./kg. Time below is in seconds, zero time being the end of the injection. Arrows in segment A indicate the beginning and end of injection. Calibrations of the arterial pressure curve, in mm.Hg. are given in segments A, C, and E.

Segment A shows the blood pressure record before, during and immediately after the dibenamine injection. At the time of onset of the injection the blood pressure record is distorted apparently because of partial occlusion of the needle against the wall of the artery. During the injection a slight rise in pressure is seen followed by a fall, the latter associated with a tachycardia. The blood pressure curve begins to recover during the injection and remains stabilized during the minute after injection recorded in this segment.

Segment B is a portion of the continuous record taken 7 minutes after the end of injection. It shows further recovery of the diastolic, systolic, and pulse pressure and the heart rate.

Segment C, taken from the continuous record 33 minutes after the end of injection, illustrates the depressor effect following evidences of discomfort. The drop in pressure at the beginning of C and the more sustained one following the calibration were initiated by struggling.

Segment D, taken 8 minutes after C, shows the trend to recovery from the second protracted depressor effect.

Segment E, taken 51 minutes after the end of injection, shows the return of blood pressure to its stabilized level. The interruption of the blood pressure curve in the middle of the segment is due to the inadvertent displacement of the manometer needle from the artery. The attempts to reinsert the needle led to a depressor effect as revealed by the lower level of the blood pressure when the needle was reinserted. The return to the established level is shown towards the end of segment E.

This figure therefore illustrates the depressor action of dibenamine and its tendency to reverse the blood pressure effects of minor trauma and emotional upsets which we ascribe to a reversal of the effects of endogenously produced epinephrine. Discussed further in the text

kilo) was given more slowly, the side actions as well as the blood pressure effect were absent.

Propylene glycol, (2 to 4 cc.) used as a solvent for dibenamine in the early ex-

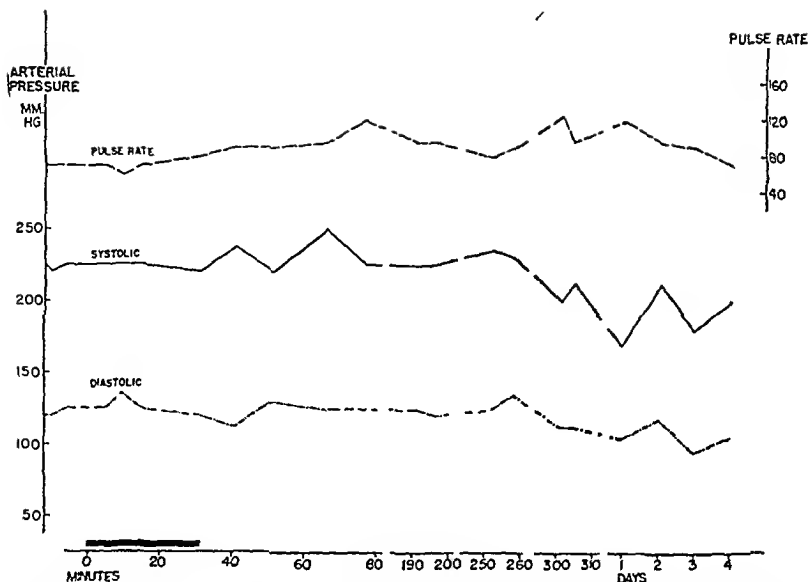


FIG. 2. Graph illustrating a marked fall in blood pressure obtained in dog Z-20 following a single dose of dibenamine intravenously 20 mgm/kg. The horizontal block at the bottom indicates the time of injection. The pulse rate in beats per minute and the blood pressure in millimeters of mercury. Time below in minutes and days. Zero time is the beginning of injection. Discussed further in text.



FIG. 3. Graph illustrating a marked fall in blood pressure obtained in Z-23 following a single dose of dibenamine intravenously 20 mgm/kg. Conventions as in fig 2. In this animal it took 26 hours for the diastolic pressure to return to its control level. As in fig 1, an initial pressure rise occurred, in this instance just after the injection period. Discussed further in text.

periments, had no such effects on the blood pressure and heart rate and was without the untoward side actions in the six dogs tested.

*Effect of repeated dibenamine injections on the blood pressure.* Dibenamine in the dosage of 20 mgm./kilo was given to four Goldblatt hypertensive dogs at 3 day intervals over a 3 week period. This treatment led to no sustained fall in blood pressure in any of the animals. Three of the animals showed a reduction in blood pressure only on the days the injections were given. In the fourth animal there was no change in blood pressure even on the days of injection. The same side actions seen in earlier experiments occurred with these maintenance

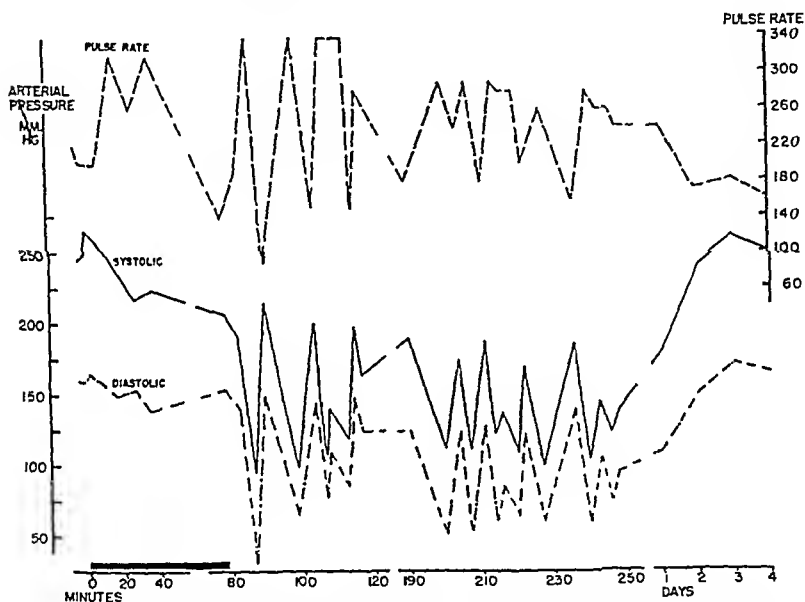


FIG. 4. Graph illustrating the lowering and marked instability of blood pressure obtained in Z-18 following a single dose of dibenamine intravenously 30 mgm/kg. Conventions as in fig. 2. Discussed further in text.

doses of dibenamine. (In one animal thrombosis of the injected vein developed with venous insufficiency evidenced by marked pitting edema.)

*Effect of dibenamine on the blood pressure response to epinephrine.* Epinephrine was used in the dosage of 1 mgm. intravenously. In 5 of 7 animals dibenamine reduced the pressor response to epinephrine and in 3 of the 7 animals it increased the magnitude of the depressor phase (table 2). These changes were most conspicuous the first 48 hours but tended to persist in some animals to a lesser extent over the next few days. Complete elimination of the initial pressor phase with a classical exhibition of the depressor response was seen when epinephrine was given two hours after the administration of dibenamine (figs. 5 and 6).

*Effect of dibenamine on epinephrine induced tachycardia.* The data on the effects of dibenamine on epinephrine-induced ventricular tachycardia are summarized in table 3. The methods used in the experimental production of paroxysmal ventricular tachycardia are considered in another communication concerning the tachycardia-inhibiting action of atropine (4). It was found that dibenamine prevented or shortened the paroxysms of epinephrine-induced ventricular tachycardia. This protective effect lasted for several days.

**DISCUSSION.** Our results confirm the view that dibenamine has an adrenolytic action. This action appears in the unanesthetized animal. However, the dura-

TABLE 2

*Effect of Dibenamine on the blood pressure responses to 1 mgm. Epinephrine intravenously as compared to control Epinephrine responses*

ANIMAL	EFFECT ON PRESSOR PHASE	EFFECT ON DEPRESSOR PHASE
Z-6	0	Increased
Z-38	0	0
X-56	Reduced	Increased
Z-18*	Reduced	0
Z-20	Reduced	0
Z-23	Reduced	0
Z-31	Reduced	Increased

\*Both 20 and 30 mgm/kilo were used in this animal with identical results.

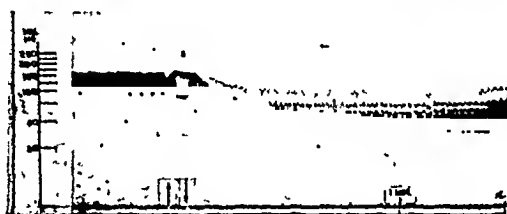


FIG. 5. Illustrates the blood pressure response to 1 mgm. epinephrine injected intravenously 2 hours after the administration of dibenamine intravenously 30 mgm./kg. Arrows indicate the onset and end of the epinephrine injection. Conventions as in fig. 1. The depressor response is obvious. Discussion in text.

tion and the constancy of the blood pressure fall following single or repeated doses of dibenamine was variable in our animals. Sometimes the effect was absent and sometimes it was extremely marked, apparently independent of the dosage. It may have depended in some measure on the rate of injection. Occasionally the action in the same animal was variable but there was a more striking variation between different animals, some exhibiting greater sensitivity. When a drop in blood pressure occurred, it lasted from 1 hour to 2 days, with a progressive diminution of the effect towards the end of this period. Dibenamine had no greater effect on the Goldblatt hypertensive dog than on the normotensive dog. It does not appear to be an effective anti-hypertensive agent. Actually

the extreme variability of the blood pressure after the exhibition of dibenamine was disadvantageous as was the short duration of the depressor effect when it occurred.

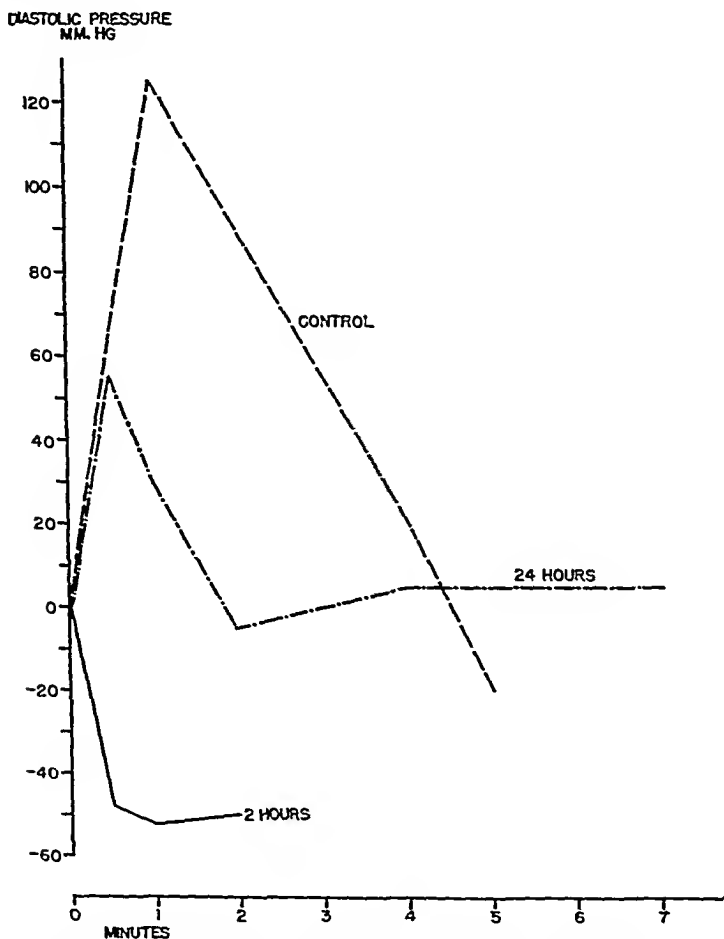


FIG. 6. Graphs illustrating the responses of the diastolic pressure to 1 mgm. of epinephrine administered intravenously at various times before and after the intravenous administration of dibenamine 30 mgm./kg. The ordinates show millimeters of mercury for the blood pressure, zero representing in each instance the diastolic pressure before epinephrine administration. Abscissae in minutes.

The variability in the blood pressure following dibenamine may give an erroneous idea of the blood pressure lowering action if readings are taken at infrequent intervals. This is true particularly under the conditions of our experi-



ments since we have found on several occasions that excitement or the process of inserting the needle into the artery of these unanesthetized animals causes a fall instead of the expected rise in blood pressure (fig. 1). This may be due to the liberation of endogenous epinephrine, with inhibition of its usual pressor response and the consequent appearance of the usually occult depressor response.

The sinus tachycardia which accompanied the temporary reductions of blood pressure was probably reflexogenic, presumably operating through the carotid sinus and root of the aorta end organs, in compensation for the depressor effects.

We have confirmed that dibenamine decreases the pressor effect of epinephrine and augments its depressor action. In our experience in the dog the maximal inhibition of the pressor response to epinephrine can be elicited early (within 2 hours) after giving dibenamine. Within 24 hours, with the weakening of the dibenamine action, the pressor action becomes apparent and within 3 to 4 days

TABLE 3  
*Effect of Dibenamine on Epinephrine-induced ventricular tachycardia*

DOG	BLOOD PRESSURE STATUS	DOSAGE OF DIBENAMINE USED  mgm./kilo	DURATION OF VENTRICULAR TACHYCARDIA						
			Before Dibenamine	After Dibenamine					
			Control	1st day	2nd day	3rd day	4th day	5th day	8th day
			sec.	sec.	sec.	sec.	sec.	sec.	sec.
Z-6	H.—1 mo. duration	20	76		19	0	2	16	
Z-38	H.—4 mo. duration	30	55 318	0	41	24	24		
X-56	H.—4 yrs. duration	20	0	0	0	0			
Z-18	H.—4 yrs. duration	20	74	0		0	0	0	
Z-20	H.—4 yrs. duration	40	121	0	0	0	0		38
Z-23	N.	20		0	0	28	48	36	
Z-31	N.	20		0	0	35	74	82	

H. = Hypertensive.

N. = Normotensive.

it assumes its normal magnitude. In general, no major qualitative differences are seen with either 50 gamma or 1 mgm. of epinephrine.

Our results regarding the inhibitory action of dibenamine on ventricular tachycardia were consistent, and in accord with previous work (2, 5, 6). The drug prevented the occurrence of epinephrine-induced paroxysmal ventricular tachycardia for several days. It suggests the possibility of the prophylactic use of this drug in the clinic after the question of dosage in relation to toxicity has been determined.

From the point of view of the pathogenesis of hypertension our results with dibenamine confirm our previous work to the effect that epinephrine has little to do with the hypertensive mechanism in nephrogenic hypertension. We previously reported (1) that epinephrine had a similar pressor action in normotensive and hypertensive animals and that in both, the use of another sympatholytic

agent (the dioxane derivative, F933) prevented the pressor response to epinephrine but did not affect the level of hypertension in the dog. Our results with dibenamine are in accord with this earlier work. It is of considerable interest that sympatholysis with these pharmacological agents bears many resemblances to surgical sympathectomy. There is weakness, instability of the blood pressure and so on. Further, the failure of the blood pressure to fall is similar to that seen in many cases of hypertension in man. The parallelism in individual cases between the effectiveness of pharmacological and surgical sympatholysis in man is worthy of further study.

#### SUMMARY

1. The intravenous administration of a single dose of the sympatholytic agent, N,N-dibenzyl-beta-chloroethylamine' (Dibenamine), to both Goldblatt hypertensive and normotensive dogs induced in 5 of 7 animals a reduction in blood pressure of approximately one hour to 2 days duration sometimes with widely fluctuating pressure levels. Administration of the drug for a period of three weeks caused no maintained reduction in arterial pressure in hypertensive dogs.

2. The inhibition of the pressor phase of epinephrine action, with the appearance of its depressor action was demonstrated within two hours following the injection of dibenamine. This inhibitory action was mainly dissipated in 24 hours.

3. Dibenamine tended to inhibit the occurrence of epinephrine-induced ventricular tachycardia.

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# A STUDY OF COMPARATIVE ANTIHISTAMINIC ACTIVITY OF SIX COMPOUNDS

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Since the report by Fourné and Bovet (1) in 1933 that certain phenolic ethers have the property of counteracting at least some effects of histamine, many new antihistaminic drugs have been synthesized and tested. The first to receive extensive clinical trial was Antergan (*N'*-phenyl-*N'*-benzyl-*N,N*-dimethylethylenediamine) (2, 3, 4). Since then, intensive research has been conducted to develop more potent and safer antihistaminic drugs for clinical use. These investigations have led to the development of such compounds as Benadryl ( $\beta$ -dimethylaminoethyl benzohydril ether) (5), Pyribenzamine (*N'*-(2-pyridyl)-*N'*-benzyl-*N,N*-dimethylethylenediamine) (6), Neoantergan (*N'*-(*p*-methoxybenzyl)-*N'*-(2-pyridyl)-*N,N*-dimethylethylenediamine) (7, 8), and Hetramine (*N'*-benzyl-*N'*-(2-pyrimidyl)-*N,N*-dimethylethylenediamine) (9). In addition, antihistaminic potency of a high order has been claimed for two new compounds, *N*-( $\beta$ -dimethylaminoethyl)-phenothiazine and *N*-( $\beta$ -dimethylamino- $\alpha$ -methyl-ethyl)-phenothiazine (10, 11). These latter compounds, designated as #3015 R.P. and #3277 R.P., respectively, were developed in the Rhonc-Poulenc laboratories in France. They have not been subjected to extensive clinical trial. The literature on antihistaminic drugs has recently been reviewed in detail by Feinberg (12).

In the present investigation, the six compounds<sup>1</sup> mentioned above have been tested side by side under the same experimental conditions, so that a direct comparison could be made of relative antihistaminic activity, and of relative toxicity.

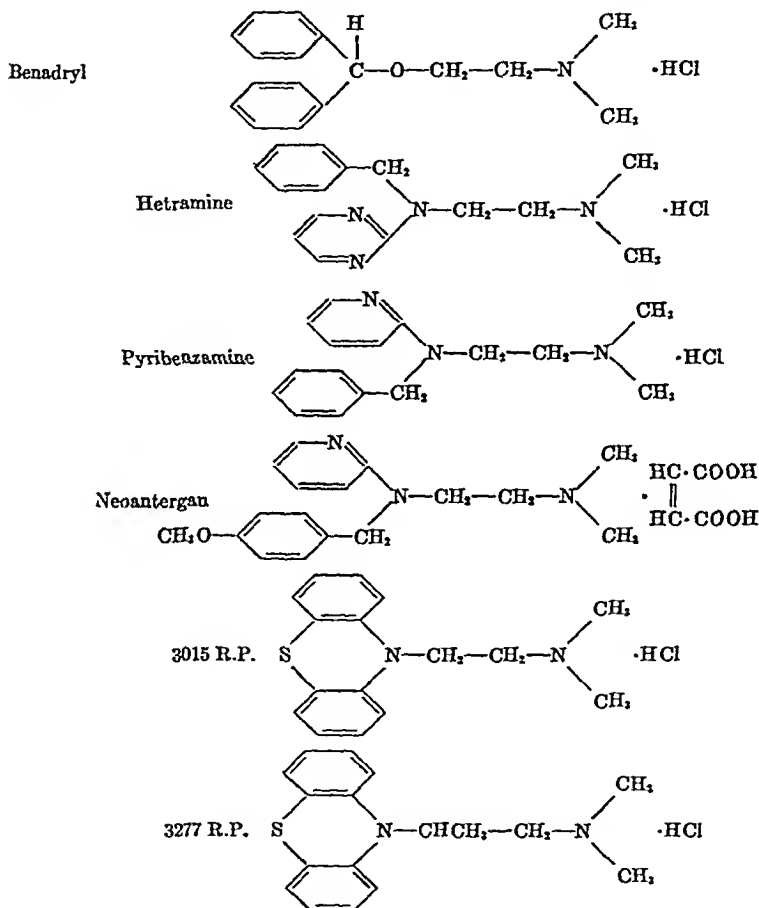
**MATERIALS.** The guinea pigs used were of a uniform Carworth strain, from 250 grams to 350 grams in weight in most of the experiments, although in the histamine aerosol experiments many had grown to double their original weight before the conclusion of the study. The mice used for toxicity determinations were of the CFI strain, and approximately 20 grams in weight. Toxicity data were evaluated according to the method of Miller and Tainter (13). The histamine used in this study was the dihydrochloride, and all doses given in this paper are in terms of that salt.

The structural formulae of the antihistaminic compounds used are shown in the accompanying diagram.

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<sup>1</sup> The compounds were obtained directly from the manufacturers, and the author gratefully acknowledges generous supplies of material from the following: Benadryl, Parke, Davis and Co.; Pyribenzamine, Ciba Pharmaceutical Products, Inc.; Hetramine, Pyridium Corporation; Neoantergan, 3015 R.P., and 3277 R.P., Société des Usines Chimiques Rhonc-Poulenc, Paris.

OBSERVATIONS. *Experiments with intravenous histamine:* The protection afforded by the compounds to guinea pigs receiving histamine intravenously was tested in two ways. In the first procedure, large doses (10 mgm. per kgm.) of the drug to be tested were injected subcutaneously, and about one-half hour



later, varying doses of histamine were administered intravenously to determine the maximum dose of histamine which the animals would tolerate. Only three of the compounds, Neoantergan, 3015 R.P., and 3277 R.P., were tested in this manner, and the results are given in table 1. It is clear that when adequately protected by an antihistaminic drug, an animal will tolerate enormous quantities of histamine, up to a thousand or more times the usual lethal dose. The number

of animals used in this experiment is too small to make an adequate comparison of potency between Neoantergan and 3277 R.P., but it is evident that both are more potent than 3015 R.P. The table includes only acute deaths from typical histamine shock. Actually, most of the "survivors" of this experiment died some hours later of perforating gastric ulcer, presumably induced by the histamine. Histamine is known to produce gastric ulcers in dogs (14), and antihistaminic drugs are of little value in counteracting the effect of histamine on gastric secretion (15) or in preventing histamine-induced ulcer (16).

In the second procedure, the quantity of histamine administered intravenously was held constant at 0.5 mgm. per kgm. of the dihydrochloride. About thirty minutes before the histamine injection, the drug to be tested was injected subcutaneously in graded doses, to determine the smallest protective dose. For this test, the animals were not re-used, for it was found in preliminary tests that an

TABLE 1  
*Acute deaths after massive histamine injections*

NEOANTERGAN, 10 MCGM./KGM.			3015 R.P., 10 MCGM./KGM.			3277 R.P., 10 MCGM./KGM.		
Histamine dosage mgm./kgm.	Number of animals		Histamine dosage mgm./kgm.	Number of animals		Histamine dosage mgm./kgm.	Number of animals	
	lived	died		lived	died		lived	died
30	2	0	25	2	0	100	2	0
40	2	0	50	1	1	160	2	0
80	2	0	75	1	1	320	1	3
160	2	0	100	0	2	400	1	1
240	1	1	160	0	2	500	1	1
320	2	2				600	0	2
400	1	1						
500	0	2						

animal which survived a near-lethal dose of histamine was partially refractory to intravenous histamine thereafter. The duration of this refractory period was not measured, for once its existence was established, it was deemed best to obtain a fresh animal for each injection.

The results of this experiment are summarized in table 2. Altogether, 311 animals were used, of which 13 were controls, receiving no antihistaminic drug. All the controls died within a few minutes of the injection, of typical histamine shock. From these results, it appears that Neoantergan is a more potent antagonist of histamine than any of the other compounds. It is followed by Pyribenzamine, 3015 R.P., 3277 R.P., Benadryl, and Hetramine, in order of potency. Three of these compounds have also been compared by Friedlander and Feinberg (17). Although they used a slightly different method for evaluating the drugs, our results are in substantial agreement with theirs.

*Experiments with histamine aerosol:* The method used for testing the efficacy of antihistaminic drugs against histamine aerosol differed from that used by Loew and associates (5). A chamber about one foot high and one foot in diame-

TABLE 2  
Protective effect of antihistaminic drugs against histamine  
0.5 mgm./kgm. intravenously

ANTIHISTAMINIC		SEVERITY OF SYMPTOMS* (NUMBER OF ANIMALS)					PER CENT SURVIVED	PER CENT SHOWING LESS THAN +++ SYMPTOMS
Kind	Dosage mgm./kgm	0	+	++	+++	death		
None (Controls)		0	0	0	0	13	0	0
Neoantergan	.03 to .06	5	2	1	2	0	100	80
	.02	1	1	3	5	0	100	50
	.01	0	2	2	6	0	100	40
	.005	0	0	0	8	2	80	0
	.0025	0	0	1	6	3	70	10
	.001	0	0	0	4	6	40	0
Pyribenzamine	.03	1	0	4	5	0	100	50
	.02	0	0	1	7	2	80	10
	.01	0	1	1	6	2	80	20
	.005	0	0	0	7	3	70	0
	.0025	0	0	1	3	6	40	10
2015 R.P.	.05	0	2	1	2	0	100	60
	.04	0	0	1	9	0	100	10
	.03	0	0	0	8	2	80	0
	.02	0	0	1	6	3	70	10
	.01	0	0	0	5	5	50	0
	.005	0	0	1	2	7	30	10
3277 R.P.	.05	0	1	1	5	3	70	20
	.04	0	0	1	7	2	80	10
	.03	0	1	3	5	1	90	40
	.02	0	0	1	3	4	50	12
	.01	0	0	0	0	10	0	0
Benadryl	.10	0	2	0	7	1	90	20
	.08	0	0	0	7	3	70	0
	.07	0	2	0	4	4	60	20
	.06	0	0	0	4	6	40	0
	.05	0	0	0	4	6	40	0
	.04	0	0	0	3	7	30	0
	.03	0	0	0	3	7	30	0
Hetramine	.40	1	0	0	3	1	80	20
	.20	0	0	1	2	2	60	20
	.10	0	0	0	2	3	40	0

\* Severity of symptoms:

0 = no symptoms.

+ = hyperpnea, restlessness, lacrymation.

++ = coughing, dyspnea, occasional gasping.

+++ = marked air hunger, convulsions, prostration.

ter was divided into four compartments with wire mesh. The lid of the chamber was made readily removable for easy access to the animals. An opening for drainage was provided in the floor. A hole in the side permitted the insertion of the nozzle of a Vaponephrin Nebulizer. Most of the droplets in the mist produced by this instrument are one micron or less in diameter (18). In operation, four guinea pigs were placed in the chamber, and a solution containing 10 mgm. of histamine dihydrochloride in 5 milliliters of water was placed in the nebulizer. Compressed air at a constant pressure of 300 mm. Hg was used to operate the nebulizer. The mist was sprayed into the chamber for a period of one minute, timed with a stopwatch. The animals were observed through a window in the side of the chamber, and graded for signs of histamine poisoning as outlined in the footnote to table 2.

It was soon found that an animal developing a "three plus" reaction would die if left in the chamber. Such animals were therefore removed, and it was found that most of them would recover promptly in room air. In this way, the same animals could be used over and over again. The reaction of a given animal to this procedure was fairly uniform in most cases, and there was no sign of refractoriness such as was observed in the guinea pigs receiving histamine intravenously. Animals repeatedly exposed to this ordeal seemed none the worse for the experience, and continued to grow in a normal fashion. The maximum time of exposure was 10 minutes. Animals which did not show a "three plus" reaction within 10 minutes of the start of the nebulization were removed from the chamber and graded according to the maximum severity of symptoms shown.

Preliminary exposures to the histamine mist were made in all animals before administration of an antihistaminic compound, and those not showing "three plus" reactions in at least three out of four preliminary exposures were discarded as insufficiently sensitive to histamine. Out of 120 animals started on this test, 93 survived the preliminary exposures, and 41 of these showed "three plus" reactions at least three times out of four. These 41 guinea pigs were accordingly used for testing antihistaminic drugs, the same animals being used for each compound. The drugs were administered subcutaneously; about one half hour was allowed between injection and exposure to the histamine mist. The experiments are summarized in table 3. It is clear that the order of potency is substantially the same as that found in the experiments with intravenous histamine. Sherrod, et al. (19) have also reported Neoantergan to be more potent than Pyribenzamine or Benadryl in counteracting bronchospasm produced by atomized histamine in guinea pigs.

It is interesting that the dosages of antihistamine drug required to protect against the aerosol were much larger than those which were fully protective against the intravenous injections. This can not be because histamine is administered in greater quantity by the aerosol method. It was found that about 0.15 milliliter of histamine solution, containing about 0.3 mgm. of histamine dihydrochloride, was nebulized in one minute. The animals actually absorbed only a small fraction of the total amount of histamine present in the chamber, but even if they had absorbed it all, it would have meant not more than 0.075

mgm. per animal. At an average weight of 300 grams, that would be about 0.25 mgm. per kgm., whereas the intravenous injection was 0.5 mgm. per kgm. The effect of a given amount of histamine is therefore greater if it is administered by way of the lungs in the form of minute droplets than if administered intravenously. A similar conclusion has been reached by others (20).

*Experiments on the isolated intestinal strip:* Histamine dihydrochloride in a concentration of 1/5,000,000 was added to Tyrode's solution in which a strip of

TABLE 3  
*Protection afforded by antihistaminic drugs against histamine aerosol*

ANTIHISTAMINIC DRUG		NUMBER OF ANIMALS SHOWING EACH GRADE OF SEVERITY OF SYMPTOMS			
Kind	Dosage mgm./2 gm.	0	+	++	+++
No drug—preliminary tests on all animals used		2	7	13	142
Neoantergan	.02	0	13	16	11
	.05	13	17	8	3
	.10	31	10	0	0
Pyribenzamine	.10	1	16	17	1
	.20	15	12	8	0
	.50	28	6	0	0
3015 R.P.	.10	0	7	17	11
	.50	18	9	8	0
3277 R.P.	.20	0	9	21	5
	.50	19	13	2	0
Benadryl	.10	0	1	17	22
	.20	0	1	21	16
	.50	0	12	18	6
	1.00	11	12	11	0
Hetramine	1.00	0	4	12	0
	2.00	4	11	3	0

guinea pig ileum was suspended. The bath was maintained at a temperature of 37°C. The histamine-induced contraction was recorded, the strip washed, and after a 10 minute rest, antihistaminic drug was added in a concentration of 1/50,000,000. Concentrations were kept the same in all experiments, so that direct comparison of potency could be made. About one minute after adding the drug to be tested, histamine was added as before. The height of each contraction was measured in millimeters, and that of the control response was taken as 100%. The "per cent inhibition" was calculated as follows:  $\frac{C - c}{C} \times 100$ ,



where  $C$  = height of control response in millimeters, and  $c$  = height of response after antihistaminic drug. Table 4 was constructed from the data obtained. The relative order of potency of the compounds was the same as in the preceding experiments

*Acute Toxicity: A. Lethal doses:* The drugs were injected intraperitoneally in mice, ten animals at each dosage level. The results, given in table 5, indicate that none of the compounds is highly toxic, compared with therapeutically effective

TABLE 4

*Activity of antihistaminic drugs in inhibiting histamine-induced contraction of the isolated guinea pig ileum; concentration of drug  $2 \times 10^{-8}$ , and of histamine,  $2 \times 10^{-7}$*

ANTIHISTAMINIC DRUG	NUMBER OF EXPERIMENTS	AVERAGE PER CENT INHIBITION OF HISTAMINE CONTRACTION
Neoantergan. . .	14	81
Pyribenzamine . .	14	68
3015 R.P.	16	52
3277 R.P..	16	45
Benadryl	13	42
Hetramine . .	12	11

TABLE 5

*Toxicity of antihistaminic drugs as tested intraperitoneally in mice*

DRUG	NUMBER OF ANIMALS	L. D. 50
		mgm./kgm.
Neoantergan . .	30	102 $\pm$ 11*
Pyribenzamine . .	50	68 $\pm$ 7
3015 R.P.	40	140 $\pm$ 13
3277 R.P.†	40	190 $\pm$ 21
Benadryl† . . . .	50	140 $\pm$ 13
Hetramine . .	40	76 $\pm$ 7

\* Standard error

† Includes delayed deaths. Delayed deaths were also observed with 3015 R.P. in one test, but these may not have been due to the drug, since the air-conditioning system in the animal room was out of order; a repetition of the test gave no delayed deaths for 3015 R.P.

tive dosage levels. However, delayed deaths, coming sometimes as late as a week after a single injection, were observed with two of the compounds, 3277 R.P. and Benadryl.

If the data in table 2 are plotted on a graph, one can calculate the approximate dosage of drug necessary to permit survival of 50% of the guinea pigs receiving intravenous histamine, 0.5 mgm. per kgm. If one divides the L.D. 50 in mice by the figure so obtained, the following therapeutic indices are found: Neoantergan 68,000, Pyribenzamine 19,400, 3015 R.P. 14,000, 3277 R.P. 9,500, Benadryl 2,333, Hetramine 500. This indicates a safety factor for Neoantergan

far surpassing that of any of the other compounds studied, although there would seem to be a wide margin of safety for all of them.

*B. Side reactions observed in mice:* Violent convulsions appeared in all groups of mice receiving 50 mgm. per kgm. or over of all the compounds. These seemed most violent in the mice injected with 3277 R.P. and 3015 R.P. The animals were observed for about a week after the injection. Within a few hours after the initial convulsions, the survivors recovered. Those surviving 100 mgm. per kgm. and over, however, appeared lethargic, the coat was ruffled, the eyes partially closed, and the animals walked with a peculiar humped-up gait. In some, there appeared to be some hemorrhage from the nares, and several had smears of coal-black fecal material in the perianal region. These reactions were especially notable in the animals receiving 3277 R.P. They were practically absent in those injected with Neoantergan or Pyribenzamine, and were of intermediate intensity in those receiving the other compounds.

**DISCUSSION.** The rationale for the use of antihistaminic drugs in the treatment of allergy rests on the observation that histamine is released during allergic reactions (literature reviewed by Feinberg (12)). While it is undoubtedly true that such release of histamine occurs in allergy, not all allergic symptoms can be accounted for in this way—for example, the relative incoagulability of the blood of dogs in anaphylactic shock. It is reasonable to assume that the allergic reaction leads to cellular damage, which in turn leads to the release from the injured cells of various substances, including histamine. In administering a drug which is specifically an antagonist of histamine, we can not hope to affect the fundamental reaction which has led to histamine release, nor can we hope to relieve any symptoms which are due to the presence of noxious substances other than histamine. It is therefore not surprising that antihistaminic drugs are not successful in every case of allergy. Such a drug can at best be a palliative for those symptoms of allergy which are referable to histamine.

A truly rational treatment for allergy would be based on knowledge of the fundamental mechanism which, among other things, releases histamine. But in the present state of knowledge, we can not predict what such a treatment would be. In the meantime, research must be aimed at (1) a better understanding of the fundamental nature of allergy, and (2) the development of the best means of treatment on the basis of what we already know. Therefore, a continued search for better and safer antihistaminic drugs is still an important problem. On the basis of the results herein reported, Neoantergan is an important addition to the armamentarium of antihistaminic drugs, and is worthy of clinical evaluation.

#### SUMMARY

Six drugs have been tested in guinea pigs for antihistaminic potency against intravenous histamine, histamine aerosol, and on intestinal strips against histamine *in vitro*. The six compounds, in descending order of potency, are as follows: Neoantergan, Pyribenzamine, 3015 R.P., 3277 R.P., Benadryl, and Hetramine. Although three of the compounds had a lower acute toxicity than Neoantergan, the toxic/effective dose ratio was highest for that compound. Side reactions

were least noticeable after Neoantergan, and most violent after 3277 R.P. Delayed deaths were observed after single injections of 3277 R.P. and Benadryl.

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# ON THE PHARMACOLOGY OF N<sub>1</sub>-PARA-CHLOROPHENYL-N<sub>5</sub>-ISOPROPYLBIGUANIDE (PALUDRINE)\*

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N<sub>1</sub>-p-chlorophenyl-N<sub>5</sub>-isopropylbiguanide (Paludrine) was synthesized by chemists at the Imperial Chemical Industries Limited, Manchester, England, during the course of a systematic exploration of biguanide derivatives (1). Clinical studies in England (2, 3), Australia (4, 5) and this country (6, 7, 8) indicate that this compound is an exceedingly promising antimalarial drug. Stated briefly these studies have shown that at apparently well tolerated doses, Paludrine exhibits causal prophylactic, suppressive and curative properties against sporozoite induced infections with *Plasmodium falciparum*, suppressive activity against sporozoite induced infections with *Plasmodium vivax*, and a high degree of curative activity against trophozoite induced infections with the latter parasite. On the basis of this clinical information, Paludrine appears to be one of the most promising and interesting therapeutic agents developed during the recent intensive investigations of antimalarial drugs carried out in this country and Great Britain.

It is noteworthy that despite rather extensive clinical use there is essentially no information on the pharmacological properties of Paludrine in either man or lower animals. Some data have been presented (2) on the levels of this drug which prevail in blood and urine subsequent to administration of therapeutic doses to human subjects. These data and a brief summary (9) of the absorption, excretion and tissue distribution of the compound in the mouse, rat and rabbit constitute the pharmacological information which is generally available.<sup>1</sup> There are no reports on the toxicology of Paludrine; apparently it was introduced into human subjects after toxicity studies in mice and rats (2).

It would appear highly desirable to have a detailed experimental evaluation of the pharmacological properties of Paludrine before this drug receives wide use under field conditions where control is difficult. Such information is also needed for a complete assessment of the relative merits of Paludrine and other antimalarial drugs. Recognizing these needs, explorations of the pharmacological characteristics of Paludrine were initiated in this laboratory in October, 1946.<sup>2</sup> The investigations included assessments of the acute and chronic toxicities of

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<sup>1</sup> A detailed report on the studies in rodents carried out by A. Spinks has been supplied us through the generosity of the author. It is our understanding that this report is to be published shortly (10).

<sup>2</sup> We are indebted to Dr. D. G. Davey of Imperial Chemical Industries Limited, Manchester, England, for the Paludrine used in this study. The compound was provided as the monohydrochloride.

the compound in mice, rats, dogs and monkeys, studies on absorption, excretion and tissue distribution in these animals and a limited examination of some of the peculiar physiological characteristics of the drug. The results of this work are reported here.

**EXPERIMENTAL. I. Acute Toxicity.** The acute oral toxicity of Paludrine has been studied in the white mouse, white rat, dog and rhesus monkey (*Macaca mulatta*). In this work the drug has been administered as the water soluble dihydrochloride. Precise toxicity evaluations have been made in the mouse; in the other species, only approximate evaluations have been made. The results of these toxicity determinations have been summarized in table 1.

The data presented show that there are substantial differences in the oral toxicity of Paludrine for different animal species. The mouse appears to be the most susceptible of the animals studied; the rat is next in order. While no toxicity ceiling has been reached in either the dog or monkey, it is apparent that Paludrine has lower acute toxicity in these species than in the mouse or rat. The LD 50 for the mouse was 23 mgm. per kgm. body weight. The lethal dose for the 150 to 200 gram rat approximated 200 mgm. per kgm. Preliminary experiments indicate that young rats are considerably less susceptible to Paludrine than older animals. Thus in the 60 gram animal the lethal dose approximated 800 mgm. per kgm. The lethal dose for the dog or monkey was in excess of 400 mgm. per kgm., this amount being without serious toxic effects.

Some comment should be offered on the reactions produced by the administration of lethal and sublethal doses of Paludrine to the various animals. In the mouse the dose-effect curve was extremely steep. Doses of 15 mgm. per kgm. failed to kill any of 20 mice. Doses of 20 mgm. per kgm. were fatal to but 4 of 45 animals, whereas doses of 25 mgm. were fatal to 14 of 20 mice and doses of 30 mgm. to 42 of 45. Mice which succumbed to Paludrine exhibited ataxia and impairment of respiration 2 to 4 hours after receiving the drug and succumbed within 4 to 24 hours. Sublethal doses were devoid of effects on the growth or general well-being of the mouse. Rats receiving lethal doses of the drug suffered marked depression and respiratory embarrassment, with death occurring within 4 to 48 hours. Sublethal doses caused a slight depression of growth in the growing rat or a slight weight loss in the older animal. Both dogs and monkeys, receiving 200 or 400 mgm. doses of Paludrine, vomited repeatedly 2 to 4 hours after the drug had been administered; this was the only immediate toxic effect which was observed. There were no delayed toxic symptoms.

The acute intramuscular toxicity of Paludrine was determined in the mouse, dog and monkey (table 1). In the mouse the LD 50 was approximately 20 mgm. per kgm., a figure only slightly less than that obtained for the oral toxicity of the drug. In the dog and monkey, however, Paludrine was considerably more toxic when given by the intramuscular route than when taken orally. In either species, a dose of 160 mgm. per kgm. intramuscularly was fatal. Death occurred between 3 and 4 hours after the drug had been injected. The symptoms of fatal intoxication were first a profound lethargy, with slowing of the heart rate and

TABLE 1

*The acute toxicity of Paludrine for the mouse, rat, dog and monkey*

ANIMAL	DOSE	NO. DEATHS PER NO. ANIMALS TREATED	REMARKS
	<i>Mgm. Paludrine base per kgm. body weight</i>		
Oral Toxicity			
Mouse*	15	0/20	Surviving mice exhibited no toxic symptoms.
	20	4/45	
	25	14/20	
	30	42/45	
Rat†	100	0/4	No toxic effects. Survivors lost slight amount of weight.
	200	3/6	
	400	6/8	
	800	4/4	
Dog	25	0/1	No toxic effects. Vomited repeatedly 2 to 4 hours after treatment.
	50	0/1	
	100	0/1	
	200	0/1	
	400	0/1	
Monkey	25	0/1	No toxic effects. Vomited repeatedly 2 to 4 hours after treatment.
	50	0/1	
	100	0/1	
	200	0/1	
	400	0/1	
Intramuscular Toxicity			
Mouse*	15	0/20	Surviving mice exhibited no toxic symptoms.
	20	9/20	
	25	16/20	
	30	19/20	
Dog	20	0/1	No toxic effects. Depression of 12 hours duration.
	40	0/1	
	80	0/1	
	160	1/1	
	320	1/1	
Monkey	20	0/1	No toxic effects. Depression of 8 hours duration.
	40	0/1	
	80	0/1	
	160	1/1	
	320	1/1	

\* The white mice used in these experiments weighed 14 to 16 grams.

† The rats used in these experiments were of the Sprague-Dawley strain and weighed 130 to 220 grams.

respiration, followed by coma. An intramuscular dose of 80 mgm. per kgm. produced a marked depression lasting for some 8 to 12 hours.

II. *Chronic Toxicity. A. Experiments with mice.* A limited study of the short-term chronic toxicity of Paludrine was carried out. In this work 5 groups of 15 white mice each (13 to 17 grams weight, with 1 gram variation within a group) were fed for 10 day periods on diets of ground Purina Dog Chow containing 0.005, 0.01, 0.02, 0.04 and 0.08 per cent Paludrine.<sup>3</sup> A sixth group of 15 animals served as controls and received the basal diet without drug. Daily observations on the outward behavior of the mice, weight changes, and food and drug intakes were recorded. Animals succumbing to treatment and selected

TABLE 2  
*Subacute toxicity of Paludrine for the mouse*

GROUP*	PER CENT PALUDRINE IN DIET	AVERAGE DAILY FOOD INTAKE	AVERAGE DAILY PALUDRINE INTAKE	AVERAGE WEIGHT GRAMS		REMARKS
		Grams per mouse	Mgm. base per kgm. body weight	Initial	Final	
Control	0	3.5	0	17.6	19.5	Fatal to 4 mice. Deaths occurred on 3rd, 6th, 9th, and 10th days. All mice dead by 10th day. Deaths occurred as follows: 2 on 4th, 2 on 5th, 6 on 6th, 1 on 7th, 3 on 8th, 1 on 10th days.
A	0.005	3.0	9	16.4	16.2	
B	0.01	2.8	18.5	15.2	15.1	
C	0.02	2.4	32	15.2	14.4	
D	0.04	1.4	45	13.7	11.0	
E	0.08	0.4	21	15.4	—	

\* Each group comprised 15 mice.

mice from groups where no fatalities occurred were examined for gross evidences of pathology. The more significant findings in this experiment are summarized in table 2.

The most prominent, if not the only, toxic effect of Paludrine was on the appetite. Animals receiving the highest levels of this drug in the diet (0.04 and 0.08 per cent) literally starved to death (table 2) and on even the lowest level (0.005 per cent) food consumption was reduced to the point where normal growth was interrupted. These effects on appetite and resulting food consumption were probably not due to an aversion of the mouse for food containing Paludrine, but more likely were the result of some specific but as yet undefined effect on the gastrointestinal tract. This explanation is indicated by the occur-

<sup>3</sup> Paludrine as the monohydrochloride was used in this and the other chronic toxicity studies.

rence of the same dramatic loss of appetite in dogs and monkeys which received the drug via stomach tube approximately 6 hours prior to feeding.

The daily dose of Paludrine which had a significant effect on food consumption of the mice was exceedingly small, 9 mgm. per kgm. body weight. It is noteworthy, in contrast to the results of acute single dose experiments, that 32 mgm. per kgm. in divided doses was tolerated without fatalities. This relation between the acute and chronic lethal doses is, of course, a good indication that the compound is not extensively localized in the body.

It must be emphasized that Paludrine produced no toxic manifestations which could be dissociated from the processes of starvation. Neither did the drug produce demonstrable lesions in the heart, lungs, liver, spleen, kidneys, stomach or small intestine.

*B. Experiments with rats.* Two experiments dealing with the chronic toxicity of Paludrine have been carried out. The first of these, a preliminary study, involving but small numbers of animals, was a short-term experiment designed solely to indicate the doses of the drug which ought to be used in the major study. In this preliminary work, 5 groups of 3 rats each (Sprague-Dawley Strain) were placed on diets of Purina Dog Chow containing 0, 0.1, 0.2, 0.4 or 0.8 per cent Paludrine. Daily observations were made of weight changes, food and drug intakes and specific toxic reactions. This experiment was interrupted at the end of 6 days, at which time all of the rats on 0.4 and 0.8 per cent diets had succumbed. The animals on the 0.2 per cent diet were in a critical state, having suffered a 36 per cent weight loss (88 to 56 grams). The rats on a 0.1 per cent diet were in good health but had gained only a fraction of the weight gained by the untreated controls—5 grams, as contrasted with 26 grams.

It must be emphasized that deaths among the above animals appeared to be due to starvation. Aside from failure to eat and consequent loss of weight there were no specific evidences of toxicity. Necropsies revealed no remarkable findings.

The major experiment was organized on the basis of this preliminary study. Six groups of rats, 9 males and 9 females per group, were used in the main experiment. These animals received diets of ground Purina Dog Chow containing Paludrine in concentrations of 0.0, 0.0125, 0.025, 0.05, 0.075 or 0.1 per cent for a period of 60 days. Observations on the general state of health of the animals, weight, and food and drug intakes were recorded daily. At the end of the 60 day period representative animals from each group were sacrificed for studies of organ pathology and the distribution of Paludrine in the various organs and tissues. The remainder of the rats were placed on the basal diet without drug to determine whether the toxic effects of Paludrine were reversible.

All but one of the animals survived the 60 day treatment period. The death of this rat, a male on the 0.075 per cent diet, occurred on the 35th day and was entirely unexplainable. Its death should probably not be attributed to the drug.

Suppression of growth was the only outward manifestation of toxicity which occurred in this study. The effects of various drug intakes on growth have been summarized in figure 1 and table 3. It should be noted that daily Paludrine



intakes of 11 to 23 mgm. per kgm. did not affect growth, but that doses of 45 mgm. exerted a significant depressing effect. The action of larger doses was correspondingly greater. The data recorded in table 3 appear to indicate that Paludrine has greater effects on male than on female animals. This difference may be merely an artifact reflecting the greater growth rate of the normal male rat.

The growth suppressing effects of Paludrine were probably entirely the result of depression of appetite. This was suggested strongly by the eating habits of the rats. Daily food intakes were fairly constant in the animals receiving the

TABLE 3  
*The effects of Paludrine on growth of the rat*

GROUP*	PER CENT PALUDRINE IN DIET	AVERAGE DAILY FOOD INTAKE	AVERAGE DAILY PALUDRINE INTAKE	AVERAGE WEIGHT GRAMS		WEIGHT GAIN IN PER CENT OF CONTROL GAIN
		Grams per rat	Mgm. base per kgm. body weight	Initial	Final	
Male Rats						
Control	0	16.7	0	79	282	100
A	0.0125	15.2	11.2	82	265	90
B	0.025	16.2	22.3	86	283	97
C	0.05	13.8	45.6	78	238	79
D	0.075	11.3	68.3	73	198	62
E	0.10	7.9	90.4	68	117	24
Female Rats						
Control	0	13.6	0	79	190	100
A	0.0125	12.8	11.3	83	190	96
B	0.025	12.8	23.5	82	184	92
C	0.05	11.9	47.4	77	170	84
D	0.075	11.9	71.0	71	158	78
E	0.10	7.7	91.3	63	107	40

\* Each group comprised 9 rats.

Control diets and those containing Paludrine in concentrations which did not affect growth. This was not the case with the animals that received the compound at levels which affected growth. At such doses food consumption was extremely irregular. The rats would eat normally for a day or two, then refuse food for a similar or longer period of time; after this a brief period of near normal food intake would ensue.

The changes in weight which occurred after withdrawal of Paludrine and restoration of a normal diet are shown graphically in figure 1. The rapidity with which growth was resumed at this dietary change clearly indicates that the effects of Paludrine were readily reversible.

It should be noted that at the doses of Paludrine employed in this study there was no evidence of organ pathology.

C. *Experiments with dogs and monkeys.* Since the experiments with these animals were performed with essentially the same techniques, they will be

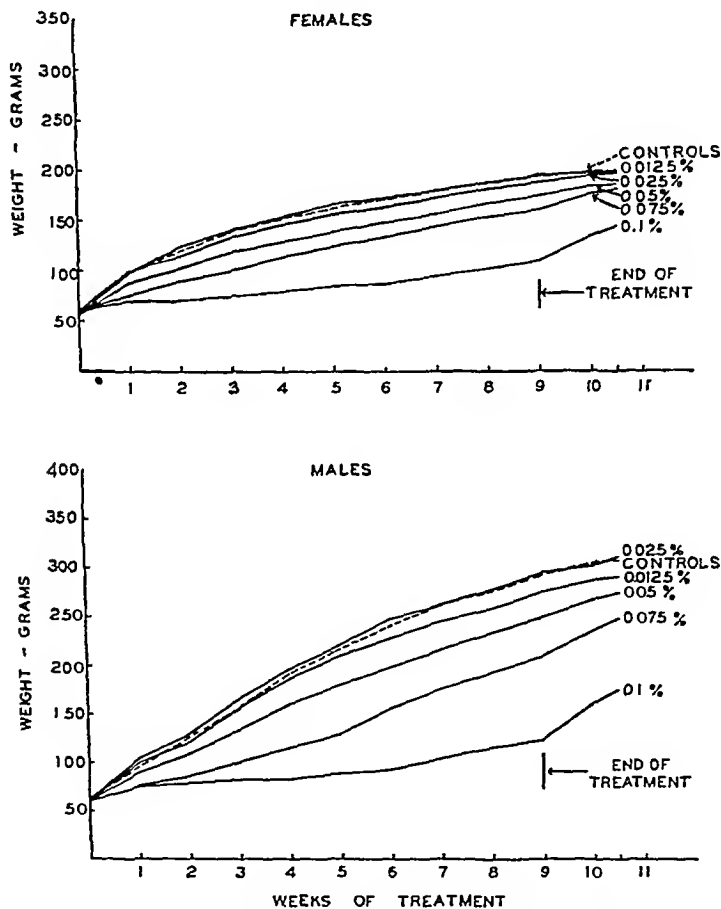


FIG. 1. THE EFFECTS OF PALUDRINE ON GROWTH OF THE WHITE RAT

described together. Five litter mate dogs, approximately 9 months of age, and 7 rhesus monkeys were used in the study. The daily doses of Paludrine for these animals were 10, 20, 40, 80 and 160 mgm. per kgm. body weight. The daily dose was administered via stomach tube in 2 equal portions at 10 A.M. and

10 P.M. Detailed hematological studies, including total erythrocyte and leucocyte counts, differential leucocyte counts and hemoglobin determinations, were carried out on 3 separate occasions prior to the start of treatment and at weekly intervals thereafter. Electrocardiographic studies were carried out on the dogs prior to treatment and at numerous intervals during drug administration. In both species plasma Paludrine concentrations were measured on the 14th, 28th, 42nd and 56th days of treatment; on each of these days blood samples were obtained just prior to the 10 A.M. dose and 2 hours thereafter. Necropsies were performed on all animals, either as death occurred during treatment, or at termination of the experiment on the 63rd or 64th day. The organs and tissues of selected animals were analyzed for Paludrine content. The principal results of the study have been summarized in tables 4 and 5.

As shown in Table 4 only the dog which received 10 mgm. per kgm. doses of Paludrine survived treatment. This animal salivated copiously about an hour after the 10 A.M. dose on the 48th day; it exhibited no other toxic reaction and was in excellent health when sacrificed on the 64th day. The dogs which received daily doses of 20, 40, 80 or 160 mgm. per kgm. succumbed after 30, 21, 10 and 7 days treatment, respectively. All exhibited the same syndrome, copious salivation throughout the entire day but especially marked immediately after treatment, vomiting with the vomitus heavily contaminated with bile, and loss of appetite leading to extreme emaciation and death. One dog, originally weighing 18.8 kgm., lost 6.2 kgm. in a 30 day period.

The results of the electrocardiographic studies on these dogs were not especially noteworthy. A marked bradycardia was present in the severely intoxicated animals. In one dog (434) inversion of the T wave occurred.

Necropsies revealed but few remarkable gross findings. All of the animals that died as a result of treatment were badly dehydrated. One animal (436) had a grossly enlarged, very congested liver. In 3 of the dogs the stomach and proximal portion of the small intestine contained abnormally large quantities of bile. In 2 of the dogs the stomach mucosa was extremely hyperemic but no hemorrhages were present.

As indicated in table 5, Paludrine was somewhat better tolerated by the monkey than by the dog. Two monkeys on daily doses of 10 mgm. per kgm. and 2 on doses of 20 mgm. per kgm. survived 63 days treatment. One of these monkeys on the 20 mgm. dose lost considerable weight. The other survivors were in excellent health at the end of the experiment although all exhibited the same type of salivation noted above in the dog. The monkeys that received daily doses of Paludrine of 40, 80 and 160 mgm per kgm. succumbed after 22, 18 and 7 days treatment. These animals salivated profusely and continuously, ate very irregularly and lost considerable weight. With one exception the necropsies failed to reveal any gross evidence of organ pathology. In this exception (S99) there was marked enlargement and congestion of the liver.

The results of the hematological studies in both dogs and monkeys can be summarized very briefly. In all of the fatal cases there was marked hemo-concentration with an increase in hemoglobin content and erythrocyte count. The

TABLE 4  
*Chronic toxicity of Paludrine for the dog*

DOG NO.	DAILY DOSE OF PALUDRINE	DAYS OF TREATMENT	BODY WEIGHT KGM.		REMARKS
	Mgm. base per kgm. body weight		Initial	Final	
437	10	64	20.8	21.0	Salivated profusely after A.M. treatment on 48th day. Aside from this episode there was no evidence of toxicity. Sacrificed on 64th day. Necropsy revealed no gross pathology.
434	20	30	18.8	12.6	Profuse salivation on 9th day and thereafter. Loss of appetite at that time. Activity normal until 23rd day. Weak and emaciated at that time. Died on 31st day. Necropsy revealed no gross organ pathology; stomach and upper portion of small intestine contained abnormally large quantity of bile; gall bladder was greatly distended.
435	40	21	15.1	10.6	Profuse salivation on 5th day, followed by loss of appetite and emaciation. Critically weak on 10th day and thereafter. Badly dehydrated; hemo-concentration marked. Vomited considerable quantities of bile-containing fluid. Died on 22nd day. No gross pathology except in stomach; the mucosa of this organ was extremely hyperemic; the stomach, duodenum, and jejunum contained large quantities of bile.
436	80	10	12.4	9.4	Profuse salivation on 5th day, followed by loss of appetite and emaciation. Frequent vomiting; vomitus contained large amounts of bile. Marked hemo-concentration. Died on 11th day. Necropsy revealed grossly enlarged liver with considerable congestion; stomach mucosa was hyperemic; stomach contained a large quantity of bile; the gastrointestinal tract was completely free of food residue.
438	160	7	11.7	8.1	Vomited repeatedly on 3rd day and thereafter. Profuse salivation on 3rd day followed by loss of appetite and rapid weight loss. Prostrated on 6th day. Died on 7th day. Necropsy revealed no gross pathology.

TABLE 5  
*Chronic toxicity of Paludrine for the monkey*

MONKEY NUMBER	DAILY DOSE OF PALUDRINE	DAYS OF TREATMENT	BODY WEIGHT KG.		REMARKS
	Mgm. base per kgm. body weight		Initial	Final	
941	10	63	7.2	7.2	Salivated to moderate degree 1 to 2 hours after each treatment, beginning on 23rd day. Otherwise there were no manifestations of toxicity. Sacrificed on 63rd day. Necropsy revealed no gross pathology.
864	10	63	7.2	7.4	Salivated to moderate degree 1 to 2 hours after each treatment, beginning on 25th day. Otherwise there were no manifestations of toxicity. Sacrificed on 63rd day. Necropsy revealed no gross pathology.
799	20	63	6.7	6.4	Salivated profusely on 17th day and thereafter. Otherwise there was no adverse reaction to treatment. Sacrificed on 63rd day. Necropsy revealed no gross pathology.
417	20	63	7.1	4.5	Salivated profusely on 16th day and thereafter. Loss of appetite on 29th day with slow but progressive weight loss following. Animal active but emaciated when sacrificed on 63rd day. Necropsy revealed no gross pathology.
891	40	22	6.6	5.2	Salivated markedly on 13th day and thereafter. Loss of appetite at that time with slow weight loss following. Prostrated on 20th day. Died on 22nd day. Necropsy revealed no gross pathology.
899	80	18	6.4	4.7	Profuse salivation on 10th day. Loss of appetite and weight from 13th day. Prostrated on 15th day. Marked hemo-concentration. Died on 19th day. Necropsy revealed grossly enlarged liver, greatly distended gall bladder, otherwise nothing remarkable.
818	160	7	5.2	4.3	Profuse salivation on 3rd day. Loss of appetite on 3rd day. Prostrated on 5th day. Marked hemo-concentration. Died on 8th day. Necropsy revealed no gross pathology.

maximum hemo-concentration occurred in dog 436 in which the hemoglobin level rose from 12 grams per 100 ml. prior to treatment to 19 grams at time of death. There were no significant alterations in total leucocyte counts and differential counts in either dogs or monkeys. There was a normal ratio of erythroid to myeloid elements in the bone marrow with no evidence of either hyperplasia or hypoplasia.

Since Fairley's studies in humans (5) suggested that Paludrine might induce renal injury, bladder urine obtained from each of the dogs and monkeys at necropsy was examined carefully. Microscopic findings were negative; there was no albuminuria.

Additional experiments were carried out with 2 dogs to determine whether the toxic effects of Paludrine were readily reversible. One of these dogs (441) received Paludrine in daily doses of 80 mgm. per kgm., the other (442) 160 mgm. per kgm. Treatment was continued until both animals were critically ill, 10 days in the case of dog 441, 5 days in the case of dog 442. At these times it was felt that unless treatment was stopped death would ensue shortly. The improvement upon withdrawal of Paludrine was dramatic. Salivation ceased within 36 hours. Appetite returned at about that time. Both animals had suffered considerable weight loss during treatment. The weight of dog 441 had decreased from 11.4 to 8.8 kgm., that of dog 442 from 11.1 to 8.6 kgm. Ten days after treatment had been stopped these animals weighed 10.0 and 10.1 kgm. respectively; at 30 days their weights were 13.3 and 12.4 kgm. These observations clearly indicate that the toxic effects of Paludrine in the dog are readily reversible. This is in agreement with the findings obtained in the rat.

*III. Absorption and Tissue Distribution.* The method employed for the determination of Paludrine throughout experiments described in this and subsequent sections is an adaptation of the procedure described originally by Spinks and Tottey (11, 12). Some of the modifications were developed by Eichelberger (13), others by Hughes (14). Stated briefly the modifications developed in this laboratory included the following: (1) a technique for limiting the oxidation of the *p*-chloroaniline which is liberated during acid hydrolysis of Paludrine; and (2) substitution of *N*-(1-naphthyl)-ethylenediamine dihydrochloride (15) as the coupling agent in place of *N*-sulphatoethyl-*m*-toluidine used by Spinks and Tottey. As will be brought out in a later publication, the modified method yields quantitative recoveries when Paludrine is added to tissue homogenates, stomach and intestinal contents, feces, urine, and various body fluids of normal control animals.

*A. Absorption of single doses.* A preliminary study of the absorption of Paludrine has been carried out in the rat, dog and monkey. In this work the drug was administered via stomach tube as the monohydrochloride. The dose in each instance was 40 mgm. base per kgm. body weight. The animals were sacrificed at intervals after ingesting the drug. The entire gastrointestinal tract was excised and the contents thereof, pooled with such fecal material as had been obtained in the interval, were extracted and analyzed for Paludrine content.

The recovery of Paludrine from the gastrointestinal tracts of rats, sacrificed

$\frac{1}{2}$ , 1, 3 and 24 hours after treatment, amounted to 78, 69, 50 and 15 per cent of the total drug which had been ingested. Thus it would appear as if at the dose given, absorption of Paludrine by the rat is fairly complete but not particularly rapid. Absorption in the dog and monkey was not markedly different from that just described for the rat. After a 3 hour absorption period 40 per cent of the drug administered was recovered from the gastrointestinal tract of the dog, 56 per cent from that of the monkey.

*B. Concentrations of Paludrine in the plasma.* The levels of Paludrine in plasma were determined following ingestion of single oral doses of 12.5 and 25 mgm. per kgm. by dogs, and 12.5, 25 and 50 mgm. per kgm. by monkeys. As in the preceding experiments the drug was administered as the monohydrochloride via stomach tube. Blood samples were obtained at 1, 2, 4, 8 and 24

TABLE 6  
*The concentrations of Paludrine in the plasma following administration of single oral doses to the dog or monkey*

ANIMAL NUMBER	DOSE OF PALUDRINE	$\gamma$ PALUDRINE PER LITER OF PLASMA				
	Mgm. base per kgm. body weight	Hours after Ingestion of Drug				
		1	2	4	8	24
Dogs						
437	12.5	315	1,035	450	270	<20
434	25	630	1,845	540	225	<20
Monkeys						
864	12.5	90	90	20	<20	<20
417	25	90	260	90	45	<20
891	50	135	400	270	255	<20

hours. Plasma, in this and in all other blood level studies, was separated by centrifugation as soon as the blood was drawn.

As shown in table 6, the concentrations of Paludrine in the plasma rose to a peak approximately 2 hours after ingestion of the drug. Levels then declined fairly rapidly and at 24 hours were less than the minimum concentration which can be detected by present analytical procedures. Dose for dose, the plasma levels of Paludrine in the dog were much greater than those in the monkey, the difference at the peak period being 7 to 11 fold. In both dogs and monkeys, the concentrations of Paludrine bore a direct relation to the dose of drug administered.

As was mentioned in Section II of this report, plasma levels were determined approximately every 2 weeks during studies on the chronic toxicity of Paludrine for dogs and monkeys. The results of these determinations have been summarized in table 7. It should be noted that, except when the experiment terminated fatally, there was no indication of the accumulation of this drug in the plasma. In fatal cases a very striking increase occurred just prior to death.

In most instances plasma levels were considerably higher in dogs than in monkeys receiving identical doses. This difference was not as great, however, as in single dose experiments. Although for the most part Paludrine concentrations in plasma were directly related to the dose of drug, marked variations were noted in the levels of individual animals. At the peak period these individual variations never exceeded 100 per cent; at the trough period, however, the variations were as great as 500 to 600 per cent.

TABLE 7

*The concentrations of Paludrine in the plasma of dogs and monkeys receiving repeated doses of this drug*

ANIMAL NUMBER	DAILY DOSE OF PALUDRINE	$\gamma$ PALUDRINE PER LITER OF PLASMA								Terminal
		Peak levels (2 hours after dosage)				Trough levels (12 hours after dosage)				
		Weeks of treatment				Weeks of treatment				
		2	4	6	8	2	4	6	8	
Dogs										
437	10	1,035	875	750	905	50	250	83	44	4,500 3,300
434	20	1,665	875	—	—	475	834	—	—	
435	40	1,890	—	—	—	810	—	—	—	
436	80	3,210*	—	—	—	1,540*	—	—	—	
Monkeys										
941	10	334	540	625	540	42	125	165	250	7,170 4,250 10,700
864	10	625	335	420	420	124	125	83	125	
799	20	490	959	580	500	165	290	375	417	
417	20	420	875	540	540	420	500	125	210	
891	40	875	—	—	—	710	—	—	—	
899	80	1,170	—	—	—	335	—	—	—	
818	160	—	—	—	—	—	—	—	—	

\* Plasma levels after 7 days treatment.

C. *Concentrations of Paludrine in the tissues.* The tissue distribution of Paludrine was determined as part of the chronic toxicity study, described in Section II above. Representative data obtained in the rat, dog and monkey have been summarized in table 8.

The data on the rat suggest that the pattern of tissue distribution varies with the dose of Paludrine. At the lowest dose there was comparatively little difference in the concentrations of this drug in any tissue except blood plasma. Cerebral cortex, with 0.28 mgm. per kgm., had the lowest concentration, while heart muscle, with 0.60 mgm. per kgm., contained the greatest concentration. At the highest dose, the concentrations of Paludrine in different tissues varied widely. Brain still contained the lowest concentration, but the levels in erythrocytes, kidney, heart muscle, spleen, lung and liver were respectively 3.5, 5.5, 6.5, 7, 9.5 and 31 times greater than those in brain.



The data in dog and monkey indicate that the tissue distribution of Paludrine in these two species is essentially the same but differs in several respects from the tissue distribution of the drug in the rat. In the first place, at the same dose the tissue concentrations were much greater in the dog and monkey than in the rat. Secondly, localization of the drug in the liver was much greater in the former species than in the rat. Thus, on daily doses of approximately 10 mgm., rat liver contained but 0.4 mgm. Paludrine per kgm. whereas dog and monkey liver contained 22.9 and 41.3 mgm., respectively. The third difference was in the distribution of the drug between erythrocytes and plasma. In the dog and

TABLE 8

*The concentrations of Paludrine in the tissues of the rat, dog and monkey*

ANIMAL	DAILY DOSE OF PALUDRINE  Mgm. base per kgm. body weight	DAYS OF TREATMENT	PALUDRINE CONCENTRATION									
			$\gamma$ per liter		Mgm. per kgm.							
			Plasma	RBC	Brain	Cord	Lung	Heart muscle	Skeletal muscle	Liver	Kidney	Spleen
Rat*	11	60	67	380	0.28	—	0.48	0.60	—	0.40	0.41	0.40
Rat*	23	60	125	—	0.56	—	3.20	1.27	—	1.51	0.88	0.54
Rat*	47	60	266	—	0.47	—	3.53	2.70	—	2.35	1.33	1.12
Rat*	71	60	199	—	0.83	—	4.54	3.56	—	5.19	2.58	2.70
Rat*	91	60	533	3,670	1.05	—	9.82	6.41	—	31.70	5.60	7.26
Dog 437†	10	64	500	1,375	3.46	3.89	7.13	4.74	1.60	22.90	6.46	9.34
Monkey												
864†	10	63	460	—	1.13	1.29	3.70	1.69	0.72	41.30	4.03	8.14
799	20	63	960	2,100	2.42	2.72	12.32	3.82	0.83	63.80	9.16	13.21

\* Paludrine was administered in the diet to these rats.

† Paludrine was administered to these animals via stomach tube, one half the daily dose at 12 hour intervals. Dog 437 was sacrificed 4 hours after the last treatment. Monkeys 864 and 799 were sacrificed 3 and 4 hours respectively after the last dose of drug.

monkey the concentration in the erythrocytes was approximately  $2\frac{1}{2}$  times that in plasma; in the rat the ratio was 6 to 7.

It should be mentioned that the tissue distribution of Paludrine has also been studied in rats which received the drug for 14 days. Tissue concentrations in these animals were essentially the same as those in rats which received 60 days treatment. This shows that the continued ingestion of Paludrine, at sublethal doses, does not lead to accumulation of the drug in the tissues.

IV. *Physiological Disposition Of Paludrine.* A. *Balance experiments.* The excretion of Paludrine in the urine and feces and its retention in the various tissues has been studied in the white rat. In this work, 6 mature rats were given daily doses of Paludrine (monohydrochloride) equivalent to 25 and 50 mgm. base per kgm. body weight. These doses divided into 2 equal portions were administered via stomach tube at 10 A.M. and 10 P.M. for 14 consecutive

days. Urine and feces were collected separately. Prior to administration of the drug, daily collections of excreta were analyzed to make certain that the urine and feces did not normally contain substances which would be measured by the method of determining Paludrine. The first collection of urine and feces during treatment covered a period of 36 hours; subsequent collections were made at 24 hour intervals. Two rats, one on each dose of drug, were sacrificed at 12 hours, 2 at 60 hours and 2 at 108 hours after the last treatment; excreta were collected during these post-treatment periods. The amounts of Paludrine remaining in the gastrointestinal tract and the concentrations in the various tissues were determined when the rats were sacrificed.

The data on elimination of the drug in the excreta have been summarized in table 9. These show that from 30 to 40 per cent of the total daily dose of Palu-

TABLE 9  
*The excretion of Paludrine by the rat*

RAT NO.	MG. OF PALUDRINE INGESTED DAILY	PER CENT OF DAILY DOSE OF PALUDRINE ELIMINATED IN THE FECES				PER CENT OF DAILY DOSE OF PALUDRINE ELIMINATED IN THE URINE				PER CENT OF TOTAL DOSE OF PALUDRINE EXCRETED IN URINE AND FECES DURING 14½ DAY PERIOD
		During 1st 36 hours	From 1st 36 hours to 14½ day			During 1st 36 hours	From 1st 36 hours to 14½ day			
			Minimum	Maximum	Average		Minimum	Maximum	Average	
Daily dose = 25 mgm. per kgm.										
1	4.66	15.2	27.4	45.2	38.8	2.8	1.2	4.2	2.3	39.4
2	4.60	15.5	25.9	40.6	33.7	3.9	1.0	3.8	2.7	35.2
3	4.00	19.7	23.4	46.4	34.5	1.2	1.8	3.1	2.5	35.8
Daily dose = 50 mgm. per kgm.										
4	9.50	8.7	12.3	30.7	22.7	3.0	5.4	10.5	8.2	29.6
5	9.30	14.5	28.6	38.3	34.0	1.9	1.0	3.9	2.2	34.8
6	9.90	21.5	22.6	37.7	29.3	1.5	2.3	4.8	3.9	32.5

drine was eliminated in either urine or feces during the 14 days of treatment and the 12 hour interval immediately thereafter. Of this amount by far the greatest proportion was found in the feces. In 5 of 6 rats, urinary excretion accounted for only 2 to 4 per cent of the total dose whereas 29 to 39 per cent was present in the feces. In the sixth rat (4), a considerably larger quantity (8 per cent) was eliminated in the urine; interestingly, fecal excretion of the drug in this animal was comparably reduced.

It will be noted that there were substantial variations in the daily excretion of Paludrine by individual rats. The factors controlling these variations have not been determined. It should also be noted that the proportions of drug eliminated in the feces were, if anything, less on the higher doses than on the lower doses. This observation was surprising since one would expect to find proportionately more drug excreted at the higher doses if fecal elimination was primarily the result of incomplete absorption. Secretion of Paludrine into the

intestine has been considered as a possible factor in this result. Data presented in the following section will show that much secretion does occur. Its magnitude is not such, however, to account for all of the drug recovered from the feces.

Table 10 summarizes the data on the amounts of Paludrine which were excreted and the amounts which were found in the various tissues at different periods after termination of treatment. It is clear from these data that no more than 45 per cent of the total drug administered could be accounted for. Thus some 55 per cent or more must have undergone metabolic alteration.

TABLE 10

*Summary of data on the excretion of Paludrine and the concentration and rate of elimination of this drug in the tissues*

RAT NO.	TOTAL DOSE OF PALUDRINE ADMINISTERED	PER CENT OF TOTAL DOSE RECOVERED					MGM. PALUDRINE PER KG. TISSUE AT TIME OF SACRIFICE						REMARKS
		In urine and feces			From gastro-intestinal tract at autopsy	From urine, feces and gastro-intestinal tract	Brain	Lung	Heart muscle	Liver	Kidney	Spleen	
		During treatment plus 12 hours	From 12 to 60 hours after end of treatment	From 60 to 108 hours after end of treatment									
	<i>m gm.</i>												
1	65.2	39.4	—	—	1.8	41.2	0.20	0.63	0.47	0.78	0.31	0.52	Sacrificed 12 hours after last dose of paludrine.
4	133.0	29.6	—	—	9.5	39.1	0.52	8.28	4.32	10.44	4.21	4.20	
3	56.0	35.8	0.8	—	0	36.6	0	0	0	0	0	0	Sacrificed 60 hours after last dose of paludrine.
6	138.0	32.5	1.0	—	0	33.5	0	0	0	0	0	0	
2	64.4	35.2	1.3	0	0	36.5	0	0	0	0	0	0	Sacrificed 103 hours after last dose of paludrine.
5	130.2	34.8	1.1	0	0	35.9	0	0	0	0	0	0	

The data in table 10 also show the rapidity with which Paludrine is eliminated from the tissues following termination of treatment. Tissue concentrations fell below detectable levels within a period of 60 hours. Some drug was found in the excreta in that interval but at later times (60–108 hours) none could be detected.

*B. Secretion of Paludrine into the intestine.* Experiments were carried out to determine whether Paludrine is secreted directly into the intestine. Six mature rats were injected subcutaneously with the monohydrochloride, the dose being equivalent to 40 mgm. base per kgm. body weight. Animals were sacrificed  $\frac{1}{2}$ , 1, 3, 5, 8 and 24 hours later. The gastrointestinal tract was excised; the contents thereof were removed quantitatively, pooled with feces collected in the interim and the mixture was analyzed for Paludrine content. The quantities

of drug found in the above intervals were respectively 0.9, 1.2, 2.5, 2.4, 3.4 and 3.3 per cent of the amount injected. These data show conclusively that Paludrine is secreted into the intestine; as stated previously, they also show that this secretion accounts for only a small fraction of the drug eliminated from the intestine in the balance experiments described above.

The question has been raised whether the Paludrine recovered from the intestinal contents arises directly from intestinal secretion or whether it comes from the bile. Experiments with rats and dogs with biliary fistulae have shown that comparatively little of the compound is secreted in the bile, thus indicating direct secretion by the intestinal mucosa. For example, in an experiment with a biliary fistula rat, which had received 17,600  $\gamma$  of Paludrine, subcutaneously, only 0.028 per cent of this dose (5  $\gamma$ ) was recovered in the bile in a 6 hour period. In the same time 2.3 per cent (409  $\gamma$ ) was recovered from the contents of the gastrointestinal tract. In an experiment with a biliary fistula dog, which had received 460 mgm., intravenously, 0.2 per cent of the injected drug was recovered from the bile in a 4 hour period, 1.5 per cent from the gastrointestinal tract. It should be pointed out that the results of these experiments are in essential agreement with the independent observations of Spinks (10).

*C. Metabolic fate of Paludrine.* It is clear from data presented in other parts of this report that Paludrine must undergo extensive degradation in the animal body. Considerable attention has been given to the metabolic fate and site of metabolism of the drug in the rat. Unfortunately these studies have yielded nothing but negative results up to the present time. Summarized briefly, studies of the possible excretory products of the drug have failed to indicate the presence of either Paludrine conjugates or *p*-chloroaniline in urine and feces. *In vitro* studies designed to indicate the site of metabolism have failed to demonstrate any breakdown or conjugation of the drug when it is incubated with tissue slices or homogenates. Studies on the decomposition of Paludrine by intestinal bacteria have likewise given negative results. Further work is in progress.

*DISCUSSION.* In assessing the potentialities of Paludrine it seems desirable to compare the pharmacological properties of this compound with those of quinacrine and its newer relative chloroquine. Such a comparison can be made with considerable facility for the latter antimalarials were studied in this laboratory intensively (16, 17) with essentially the same techniques as were used in the investigations of Paludrine described above.

The relative acute toxicities of Paludrine, chloroquine, and quinacrine vary considerably with the experimental animal. When administered to the mouse, either orally or intramuscularly, Paludrine is considerably more toxic than the other drugs; the difference amounts to 20 to 40 times when the compounds are given via the oral route. In the rat, however, the 3 drugs exhibit approximately the same acute oral toxicity. In the dog the acute intramuscular toxicity of Paludrine is  $\frac{1}{3}$  that of chloroquine and  $\frac{1}{2}$  that of quinacrine. In the monkey, Paludrine has approximately  $\frac{1}{2}$  the acute intramuscular toxicity of either of the other drugs.

The relative chronic toxicities of the 3 compounds also vary with the experimental animal. In short-term studies in the mouse, Paludrine is approximately 5 times as toxic as chloroquine and 10 times as toxic as quinacrine. In 60 day experiments in the rat, Paludrine has  $\frac{1}{2}$  the toxicity of the other drugs. In similar studies in the dog, the toxicities of Paludrine and chloroquine are about the same; both drugs have 2 to 4 times the toxicity of quinacrine. In the monkey, Paludrine, chloroquine, and quinacrine have essentially equal toxicity.

It might be inferred from the foregoing comparison that on the basis of toxicity Paludrine has nothing remarkable to offer over either chloroquine or quinacrine. Such a conclusion would probably be correct if drugs were selected solely on the basis of fatal doses or doses which produce severe toxic reactions. It is well recognized, however, that when a drug is to be administered over long periods of time and with little or no professional supervision, as in suppression of malaria, the type of toxic reaction induced by the compound is of great importance in determining its clinical usefulness. It is necessary, therefore, to compare the types of toxic reactions which are associated with the administration of Paludrine, chloroquine, and quinacrine.

It has been stated repeatedly in this report that Paludrine exerts some effect on the gastrointestinal tract which leads to loss of appetite and ultimately to death via starvation. This effect on the digestive tract is the principal if not the sole manifestation of Paludrine intoxication. Quinacrine also affects the gastrointestinal tract, producing vomiting and severe diarrhea; however, it does not appear to inhibit appetite. This drug also produces a state of hyperirritability, severe liver pathology and at least microscopic lesions in the heart, spleen and kidney. Furthermore, it produces intense staining of the skin, a property not common to either chloroquine or Paludrine. Chloroquine produces tremors, eye symptoms suggestive of blurred vision, depression, generalized muscular weakness not associated with inanition, and low grade injury to the liver. Chloroquine appears to have less effect on the gastrointestinal tract than the other drugs.

It is important to emphasize that the toxic reactions to Paludrine are readily reversible even when intoxication is just sublethal. This is in marked contrast to findings in severe intoxications with either quinacrine or chloroquine. Recovery is seldom complete in animals which have been severely intoxicated with these latter drugs. Rats which have developed marked but not fatal toxemias during chloroquine or quinacrine administration frequently continue to decline after termination of treatment and succumb 7 to 10 days after last receiving drug. This result should be contrasted to the rapid recovery from Paludrine intoxication (cf. figure 1).

This comparison of the toxemias induced by Paludrine, chloroquine, and quinacrine suggests that Paludrine possesses 2 distinct advantages over the other drugs. These are: (1) limitation of toxic effects to the gastrointestinal tract (probably the most easily detected of all toxic reactions); (2) the ready reversibility of severe intoxications.

It should be noted that there are significant differences between Paludrine and chloroquine or quinacrine with respect to the deposition of these drugs in the tissues and their retention after termination of treatment. Thus Paludrine is found in all tissues except blood plasma in much lower concentrations than chloroquine and quinacrine. For example in rats receiving 50 mgm. doses of Paludrine for 60 days, the levels in such organs as liver, spleen, lung and kidney vary between 2.5 and 5.0 mgm. per kgm. With similar treatment, the concentrations of chloroquine range from 1,100 to 3,700 mgm. per kgm., while the concentrations of quinacrine range from 6,000 to 17,000 mgm. Except at lethal dose levels, Paludrine concentrations in blood plasma and other tissues do not increase with prolonged treatment; such increases are very marked with both chloroquine and quinacrine, particularly in tissues other than plasma. Elimination of Paludrine from the tissues after termination of treatment is much more rapid than the elimination of chloroquine and quinacrine. Thus tissues are essentially free of Paludrine within 48 hours after the last dose is administered. In contrast to this from  $\frac{1}{2}$  to  $\frac{1}{4}$  of the chloroquine present at the end of treatment and from  $\frac{1}{3}$  to  $\frac{1}{2}$  of the quinacrine are retained in the tissues for at least a week after drug administration has been terminated. These characteristics of Paludrine may well constitute another advantage of this drug over chloroquine and quinacrine, since they should facilitate control or relief of such toxic reactions as do occur.

The preceding discussion has suggested that by virtue of its pharmacological properties Paludrine has several advantages over chloroquine and quinacrine. Although it is not the province of this paper to discuss the management of human malaria, it should be noted that Paludrine also has certain advantages over chloroquine and quinacrine as an antimalarial agent. In the first place, Paludrine has the unique characteristic among the 3 drugs of functioning as a prophylactic against mosquito-induced falciparum infections; it exhibits this characteristic when a single dose of 25 mgm. is administered 3 to 5 days after infection (5). Secondly, Paludrine abolishes the clinical attack in vivax malaria in as small a total dose as 15 mgm.; this is about  $\frac{1}{12}$  the dose of chloroquine and  $\frac{1}{35}$  the dose of quinacrine which are required for the same infection (6). Thirdly, Paludrine will cure overt falciparum malaria at a dosage comparable to that of chloroquine and smaller than that of quinacrine (2, 5, 6). Finally, Paludrine effects routine suppression of either vivax or falciparum malaria at doses which are slightly lower than those of chloroquine and considerably lower than those of quinacrine (5, 8). Since, in all these attempted uses, Paludrine exhibits a greater spread between effective and toxic doses than either chloroquine or quinacrine, Paludrine appears to offer considerable promise as a generally useful antimalarial drug.

#### SUMMARY

An investigation of the pharmacological properties of Paludrine has been carried out. This has included: (1) evaluation of acute and chronic toxicities

in the mouse, rat, dog, and monkey; (2) investigation of absorption, excretion and tissue localization; (3) explorations of pathways of metabolism. A summary of the results of this study follows.

Wide variations in the acute toxicity of Paludrine for different animal species were noted. The mouse is the most susceptible of the experimental animals included in this study. The dog and monkey are the least susceptible, while the rat occupies an intermediary position. There is an 8 to 20 fold difference between the acute toxicity of Paludrine for the mouse and that for the dog or monkey, the variation depending upon the route of administration.

There are somewhat smaller variations in species susceptibility to the chronic toxicity of Paludrine. The dog appears to be the most susceptible of the test animals; the mouse and monkey are next in order, while the rat is least susceptible.

The symptoms of chronic Paludrine intoxication in all 4 animal species are referable to an as yet undefined effect of the drug on the gastrointestinal tract. This effect results in almost complete loss of appetite leading to death via starvation. Thus far there has been no evidence of toxic effects of Paludrine on specific organs other than those of the enteric tract. Severe toxemias induced by Paludrine are readily reversible when treatment is terminated.

Absorption of Paludrine from the gastrointestinal tract is 70 to 90 per cent complete but is rather slow. The drug is localized in the tissues to a comparatively slight degree. At low intakes there is little difference in the concentrations found in various organs and tissues, other than blood plasma. At higher doses, however, Paludrine accumulates in the liver to a greater extent than in other organs. There is some species difference in the deposition of Paludrine in the tissues, the organs of the dog or monkey containing considerably larger amounts of drug than organs of rats receiving comparable doses. Paludrine disappears from the tissues with extreme rapidity when treatment is terminated.

Only a comparatively small fraction of the Paludrine ingested is eliminated in the urine. Since the drug does not accumulate in the tissues, it has been inferred that it must undergo metabolic alteration. Thus far attempts to elucidate the metabolic fate of Paludrine have yielded negative results.

The pharmacological properties of Paludrine have been compared with those of chloroquine and quinacrine and some of the possible advantages of Paludrine have been indicated.

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# THE BRONCHODILATOR ACTION OF COMPOUNDS STRUCTURALLY RELATED TO EPINEPHRINE

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An examination of the pharmacology of the N-alkyl homologues of epinephrine as described by Konzett (1) will reveal that several of these compounds possess important bronchodilator activity. In a series of experiments carried out in this Laboratory, six compounds having a catechol group have been investigated as broncholytic agents in an effort to evaluate their relative effectiveness as antiasthmatics, and to attempt some correlation of their bronchodilator activity with their chemical structure. In this communication, we have described the results obtained with these compounds in histamine-induced bronchoconstriction in guinea pigs.

**METHOD.** The fact that inhalation of a histamine solution as a finely dispersed mist easily induces bronchial asthma in guinea pigs, which can be lessened in severity by various antihistaminic agents, has been discussed at length by Kallos and Pagel (2), Schaumann (3), Neely (4), and subsequent investigators. In this series of experiments, a modification of the method described by Schaumann (3), v. Issekutz (5), and Loew (6), was used to induce experimental histamine asthma. Healthy guinea pigs, selected at random, weighing from 250 to 350 grams, were confined singly in a glass chamber of about four liters capacity. The cover of the chamber (a screw-on cap about ten centimeters in diameter) was provided with two openings, one of about two centimeters in diameter through which the histamine mist was blown, and a much smaller opening through which the excess escaped, thereby keeping the concentration of mist in the chamber more or less constant and also providing for circulation. A finely atomized mist of 0.2 per cent histaminic diphosphate solution, as produced by a standard commercial model nebulizer, was blown into the chamber. It was found that approximately 300 mm. Hg. air pressure was necessary to produce a satisfactory concentration of histamine mist. A diagram of the apparatus is shown in figure 1.

Results were recorded as minutes and hundredths, in two columns: ONSET and DURATION. ONSET equalled the time necessary to produce prominent symptoms, namely, the increase in respiratory frequency, forced inspiration etc., whereas DURATION represents the total time from exposure until asphyxial convulsions or collapse were produced. Following the control exposure, all guinea pigs were permitted to recover and rest over a two to four hour period. At the end of this time they were injected intraperitoneally with the compound under test, and fifteen minutes later subjected to the histamine mist again. Times were recorded as in the control tests. All animals exposed to the mist for six minutes or longer without evidencing symptoms were arbitrarily considered fully protected. This figure was chosen after preliminary tests indicated that asthmatic symptoms and collapse could be induced in all but rare individuals within two minutes. The few animals that required appreciably longer to evince symptoms were discarded. The procedure described has not only the advantages of permitting careful individual observations, and direct comparison of effects in each animal, but also conserves the animals for future use. As has been indicated by Kallos and Pagel (2) and Ratner (7), when given sufficient rest (3 to 4 days) between experiments, the guinea pigs did not seem to develop any tolerance or diminished sensitivity toward either the histamine or the compounds tested.

RESULTS. The six compounds tested in this series of experiments were the primary amine 1-(3',4'-dihydroxyphenyl)-2-aminoethanol (nor-epinephrine) and the N-methyl (epinephrine); the N-ethyl; the N-isopropyl (Isuprel); the N-sec. butyl homologues along with the isopropyl homologue of 1-(3',4'-dihydroxyphenyl)-2-aminoethane. Results obtained are shown in table 1. From these data it can be readily seen that Isuprel is the most effective bronchodilator in histamine-induced asthma, exceeding the activity of all other compounds. This action seems to be optimal with the N-isopropyl homologue, as can be seen by comparing the activity of the compounds in order from the primary amine to the N-sec. butyl homologue. An examination of the results obtained with com-

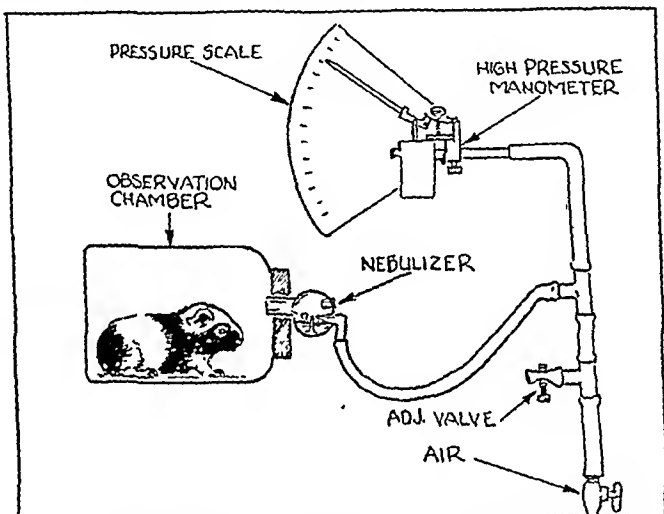


FIG. 1. SCHEMATIC DIAGRAM OF APPARATUS EMPLOYED IN HISTAMINE-INDUCED ASTHMA IN GUINEA PIGS

pound O-4, 1554 seem to indicate that the alcoholic hydroxyl on the beta-carbon is also important for the broncholytic action described here.

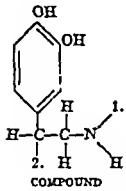
A series of lung perfusion experiments were carried out by the method of Sollman and von Oettingen as modified by Thornton (8) and the data obtained are listed in table 2. Both Isuprel and O-4, 1424 produce responses greater than epinephrine; while nor-epinephrine is distinctly less active being approximately equal to O-4, 1554. With the latter compound, the loss of the hydroxyl group on the beta-carbon causes a significant decrease in bronchodilator activity, thereby indicating the importance of this radicle.

In a few experiments, guinea pigs were sensitized to horse serum and, after a suitable interval, these animals were killed and their lungs perfused, using horse serum as the constrictor agent. The results obtained are shown in table 3.

The data presented fall into the same general pattern as established by the

TABLE 1

*Bronchodilator action of five homologues of epinephrine in histamine-induced asthma in guinea pigs*

 COMPOUND	STRUCTURE		NUMBER OF EXPERIMENTS	DOSE	CONTROL TIME*		EXPTL. TIME*		EPINEPHRINE RATIO†		TOXICITY	
	1.	2.			Onset	Duration	Onset	Dur.	Onset	Dur.	No. of mice	Approx. LD 50
				mgm./kgm.	minutes		minutes					mgm./kgm.
Nor-Epinephrine	H	OH	6	0.10	.90	1.66	1.75	2.83	52	60	22	10
1-Epinephrine	CH <sub>3</sub>	OH	6	0.10	.83	1.83	3.38	4.73	100	100	34	4
O-4, 1516	C <sub>2</sub> H <sub>5</sub>	OH	6	0.10	.82	1.58	3.20	4.80	95	102	40	27
Isuprel	CH(CH <sub>3</sub> ) <sub>2</sub>	OH	15	0.10	.83	1.43	4.91	6.00+	146	127+		
			8	0.15	.73	1.40	5.26	6.00+	—	—	203	450
			7	0.20	.66	1.24	6.00+	6.00+	—	—		
O-4, 1424	Sec. butyl	OH	11	0.10	.64	1.37	2.11	3.11	52	66	76	450
O-4, 1554	CH(CH <sub>3</sub> ) <sub>2</sub>	H	7	0.10	.59	1.23	0.90	2.00	26	42	38	490

\* Time expressed in minutes and hundredths.

† Epinephrine ratio =  $\frac{\text{Exptl. time of dilator}}{\text{Exptl. time of epinephrine}} \times 100$ .

TABLE 2

*Bronchodilator action of five homologues of epinephrine against histamine in perfused guinea pig lungs*

COMPOUND	NUMBER OF EXPERIMENTS	DOSE	CONTROL	EXPERIMENTAL	EFFECT %*
			Response to histamine	Response to histamine + dilator	
		mgm.	cc./min.	cc./min.	
Nor-Epinephrine	5	0.049	65/38	61/43	31
	3	1.4	35/17	34/33	95
Epinephrine†	6	0.005	37/18	34/32	90
	6	0.01	50/24	48/49	104
O-4, 1516	6	0.005	45/23	46/42	82
	6	0.01	45/26	44/44	100
Isuprel	6	0.005	33/14	32/36	121
	6	0.01	50/33	53/55	112
O-4, 1424	6	0.005	38/17	35/39	118
	6	0.01	39/20	41/43	110
O-4, 1554	5	1.4	44/24	44/44	100

\* Per cent Effect =  $\frac{\text{Control difference} - \text{experimental difference}}{\text{Control difference}} \times 100 = \text{per cent Protec-}$   
tion.

† 1-epinephrine was used throughout the experiments. The other compounds were racemie.

previous results. Although only three of the six compounds were tested, it would appear that the bronchodilator efficiency is of an ascending order again corresponding directly to the size of the N-alkyl group with Isuprel being the most effective. It may be inferred from the previous data that the remaining three compounds would demonstrate activity according to their specific characteristics and hence take their respective places in the activity scale.

Figure 2 represents graphically the results obtained in the histamine-induced asthma studies. The progressive increase of activity to the right is readily seen. The order of effectiveness of the compounds is according to the size of their respective N-alkyl groups, with the exception of O-4, 1554 which is placed first

TABLE 3

*Bronchodilator activity of three sympathomimetic amines in bronchial constriction produced by horse serum in sensitized perfused guinea pig lungs*

COMPOUND	CONTROL VOLUME	EXPTL. VOLUME DIFFERENCE IN %*	NUMBER OF EXPERIMENTS
	<i>cc./min.</i>		
Horse Serum only 5 mg.	45	15.5	6
Epinephrine 0.01 mg. H. S. = 5 mg.	55	105.0	3
O-4, 1516 0.01 mg. H. S. = 5 mg.	39	146.0	3
Isuprel 0.01 mg. H. S. = 5 mg.	34	182.0	6

\* Exptl. volume = Per cent of control volume; control volume equalling 100 per cent in each case.

because of its low activity. The absence of the alcoholic hydroxyl in O-4, 1554, the remainder of the molecule being identical with the most active member of the series, therefore assumes considerable importance. The apparent discrepancy between the relative activity of epinephrine and O-4, 1516 is not readily explainable as the increase in size of the N-alkyl group would lead one to assume a significant superiority of the N-ethyl over epinephrine. The last bar on the graph illustrates the activity of groups larger than isopropyl, O-4, 1424 being the N-sec. butyl homologue of epinephrine. The isopropyl member of the series exceeds the others in broncholytic activity by a considerable margin, its onset time exceeding the duration time of both epinephrine and O-4, 1516.

The comparative toxicity of the compounds was determined by intraperitoneal injection into albino mice. All the animals were from our own colony and housed under conditions of constant temperature and humidity. The mice weighed

from fifteen to twenty-two grams and were observed for seventy-two hours following injection. Only the L.D. 50 results are given in table 1. The toxicity of the N-isopropyl homologue (Isuprel) is slightly greater than that of the N-sec. butyl (O-4, 1424) and the ethane derivative (O-4, 1554). All three of these compounds are remarkably low in toxicity but it can be seen that toxicity does not correspond directly with broncholytic activity as the marked difference between the first three compounds and the latter three demonstrate. From the data shown here, it would seem that an important factor for low toxicity lies in the

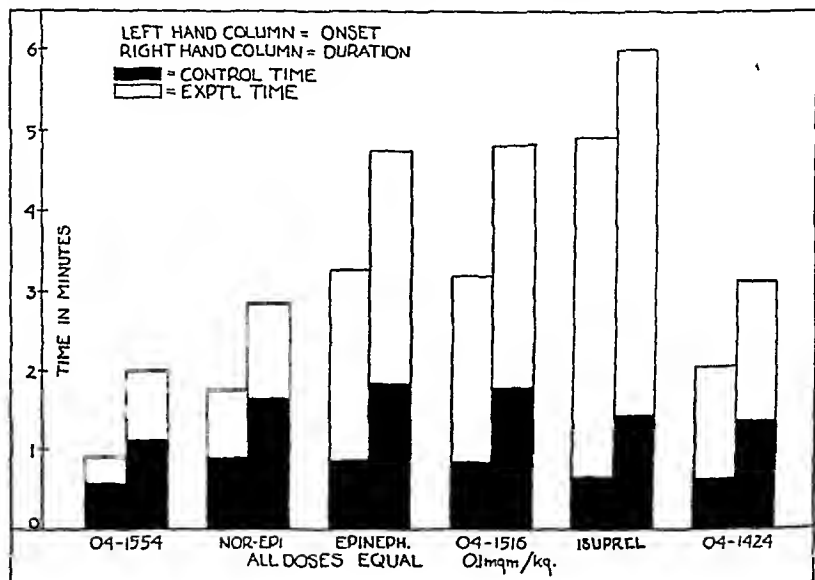


FIG. 2. ANTI-ASTHMATIC ACTIVITY OF FIVE HOMOLOGUES OF EPINEPHRINE

Time is expressed in minutes. Time for both control and experimental results is measured from zero. Left hand columns represent ONSET times, right hand column represents DURATION. All drugs were given intraperitoneally fifteen minutes prior to histamine mist exposure. Results were recorded as described under "Method".

branching of the alkyl group attached to the nitrogen, a characteristic common to the last three compounds in the table.

**Discussion.** While many workers, including Kallos and Pagel (2), Schumann (3), v. Issekutz (5), and Loew (6), have described the effects of a wide variety of compounds in histamine-induced asthma, it was Konzett (1) who first described the broncholytic activity of the N-alkyl homologues of epinephrine in detail. Although Konzett (1) states that the isopropyl homologue has approximately ten times the effectiveness of epinephrine as a bronchodilator in anesthetized dogs, our data on histamine asthma in guinea pigs do not indicate so large a difference. However, the isopropyl homologue is clearly superior in

bronchodilator activity to any other member of the series. From the data gathered in this group of experiments (tables 1 and 3), it seems that the alcoholic hydroxyl on the beta-carbon of the side chain, and an alkyl on the nitrogen, is important for bronchodilator activity of these compounds. It is noteworthy that the only member of the series not possessing the alcoholic hydroxyl is also the weakest in activity. The result of the removal of the OH group (O-4, 1554), as well as the N-alkyl, as in nor-epinephrine, therefore indicates that both of these groups contribute to the nature as well as the degree of the pharmacologic activity.

While the N-ethyl homologue (O-4, 1516) is approximately as effective as epinephrine, the sudden pronounced increase in activity with the addition of one more carbon to the N-alkyl chain, can only be explained by the branching of the chain since Konzett (1) has reported the N-n. propyl homologue to be very weak. However, the N-sec. butyl (O-4, 1424) does not show an increased activity over the N-isopropyl as might be expected. It may be inferred therefore that the N-alkyl group acts as a haptophore for a highly selective receptor mechanism. Compounds bearing groups that correspond closely to the optimal haptophore (in this case N-isopropyl), elicit responses proportionate to their degree of similarity. This suggests there are definite structural requirements for bronchodilation comparable to those which have been described as essential for vasomotor effects.

#### SUMMARY

1. The bronchodilator effect of five homologues of epinephrine is described.
2. The N-isopropyl homologue, 1-(3', 4'-dihydroxyphenyl)-2-isopropylaminoethanol, was found to be the most effective bronchodilator. The removal of the alcoholic hydroxyl from the beta-carbon, to form 1-(3', 4'-dihydroxyphenyl)-2-aminoethane, resulted in a very great reduction in activity.
3. The branching of the N-alkyl group appears to markedly reduce toxicity.
4. The structural requirements important for effective bronchodilator activity are discussed.

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# THE ACUTE TOXICITY OF THIOUREAS AND RELATED COMPOUNDS TO WILD AND DOMESTIC NORWAY RATS<sup>1</sup>

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Following the observation that phenyl thiourea has a high acute toxicity to the Norway rat (1), a systematic investigation was undertaken in this laboratory, with the purpose of finding some related compound which would serve as a practical rodenticide. Such a substance was found, namely alpha-(or 1-) naphthyl thiourea (ANTU) (2), which has proved very efficacious as a field poison for Norway rats. The present report summarizes the toxicity data obtained in the course of the screening, which was first performed with laboratory rats and later extended to recently trapped wild Norway rats.

**MATERIALS AND METHODS.** None of the compounds used was synthesized in this laboratory: of the 196 compounds here reported, 127 came from E. I. du Pont de Nemours and Company, 30 from the American Cyanamid Company, and the remainder from various other sources, as noted in the tables. These compounds were used as received: they were not recrystallized and no attempt was made to evaluate their purity. Most of the samples were crystalline powders, notable exceptions being thioformamide and some of the aromatic amines.

The 850 adult wild Norway rats on which data are included in the tables were trapped for us by the Baltimore City Rodent Control Office. Each substance was administered to them according to a standardized technique, reported elsewhere (3): unanesthetized animals, restrained in a "sock", received doses of the various substances, suspended (or dissolved) by mortaring in a 10% solution of gum acacia in water. The dose was given through a metal stomach tube in a total volume graduated to the rat's body weight. The rats received no food the night before dosing; their regular diet of purina fox chow was returned to them after poisoning. In general, any rats which had not died within 4 to 7 days were discarded and counted as survivors.

Autopsies were performed on all animals which died. Every effort was made to use only healthy specimens; owing, however, to the relatively high incidence of pathologic conditions in wild rats, autopsy occasionally revealed evidence of disease sufficient to have caused death or at least extreme debilitation. Data on such rats are not included in the tabulated values. To get around the difficulties inherent in the possibility that a whole group of rats might prove to be in poor physical condition, no more than 4 of the wild rats used in the assay of any given compound were dosed on the same day. The rats were used as they came, without regard to sex, but in general there were both males and females in each assay.

The 789 adult domestic Norway rats came from various sources and had occasionally been previously used for other purposes which in no way interfered with their susceptibility to drugs. Administration of the compounds was in most cases by stomach tube in propylene glycol, although sometimes the intraperitoneal route was used. The food provided for these rats was usually purina fox chow.

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<sup>1</sup> Started under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and The Johns Hopkins University and continued under a contract between the Medical Division, Chemical Corps, U. S. Army, and The Johns Hopkins University.

The domestic rat assays were performed by several different people, over a period of three years, while the wild rat assays were done almost without exception by one person, and later checked by one other person, during the months May 1946 to January 1947.

*Arrangement of the tables.* The various compounds<sup>2</sup> are grouped into tables according to their chemical structures, beginning with those substances most closely related to thiourea and proceeding through compounds which by successive substitutions contain less and less of the full thiourea configuration. Tables 5 and 6 contain miscellaneous compounds which were available for assay but which bear no particular structural relation to thiourea.

Under each subdivision of a table the compounds are listed in order of decreasing toxicity to wild Norway rats (increasing LD50's or acute median lethal doses), or of decreasing toxicity to domestic rats if no wild rat assays were made. The samples available were frequently small and therefore some assays are not as complete as we should have preferred.

Unless an assay on domestic rats had been performed previously, giving an indication of the dose range to be tried, each assay on wild rats was begun with four rats at 100 mgm./kgm., and if none of these rats died no further tests were usually made. A notation in the LD50 column of 100+ corresponds to this result.

The number of rats included in each assay was not in general large enough to justify computation of statistical limits of error; standard errors will therefore be found only for a few compounds (in table 1). These standard errors were estimated by the method of Litchfield and Fertig (4), using logarithmic-probit graph paper. No attempt was made to calculate a standard error unless data on at least 12 rats fell between probits 4 and 6. In all other cases, therefore, the accuracy can be judged only by the relative number of rats used. LD50's followed by a question mark are considered to be relatively less accurate than the number of rats used would otherwise indicate. A minus sign as occasionally used before an LD50 for domestic rats indicates that the dose listed was the lowest given and killed more than 50% of the animals.

**RESULTS.** Table 1 lists the toxicities of 29 compounds (including thiourea itself) which contain the thioureido grouping; in which only a single hydrogen of the thiourea molecule is replaced by a substituent. Of this number 12 proved to have LD50 values for wild Norway rats below 100 mgm./kgm., and of these 11 contained a benzene ring directly attached to the thioureido radical. Each of these compounds which was assayed on domestic rats was toxic to them as well, but in addition thiourea itself and two aliphatic derivatives were toxic to domestic rats although relatively nontoxic to the wild rats.

Table 2 includes 40 disubstituted thioureas, all of which contain the thioureylenic grouping either in straight chain or as part of a five or six membered ring. None of these compounds exhibited high toxicity either to wild or domestic rats: the most toxic were N-n-butyl-N'-phenyl thiourea and 5-chloro-2(3)-benzimidazolethione, each of which had an LD50 of 150 mgm./kgm.

Table 3 contains 7 compounds in which 2, 3 or 4 of the hydrogens of thiourea have been replaced by substituents. Only wild rat assays were performed on these substances, and none proved to have high toxicity.

Table 4 shows the results obtained with 5 thiopseudoureas (isothioureas), 7 guanidines, 12 thioamides, and 11 thiazole derivatives. The thiopseudoureas

<sup>2</sup> An effort has been made in naming the compounds to use a consistent system and one which is in accordance with the principles outlined in the 1945 index to Chemical Abstracts. In table 1, the N- which should precede most individual names has been omitted to save space, as has the N, N' in table 2.



of necessity no longer contain a sulfur atom doubly bound to a carbon, and may be considered as thioureas which have been forced to remain in the thioenol form by virtue of substitution on the sulfur atom. Again none of these compounds

TABLE 1  
*Thiourea and singly N-substituted thiourea derivatives*  
(All contain thioureido grouping:—NHCSNH<sub>2</sub>)

NAME	FORMULA	SOURCE	WILD NORWAY RATS		DOMESTIC RATS	
			LD 50	No used	LD 50	No used
			mgm /kgm		mgm /kgm	
<b>A. Parent Compound</b>						
Thiourea (See ref (5))	NH <sub>2</sub> CSNH <sub>2</sub>	M	1830		1.25 to 640	
<b>B. N-Alkyl Thioureas</b>						
Ethyl thiourea	CH <sub>3</sub> CH <sub>2</sub> NHCSNH <sub>2</sub>	K	100	8	—	
Acetyl thiourea	CH <sub>3</sub> CO NHCSNH <sub>2</sub>	EK	100+	8	50	4
4-Morpholinylmethyl thiourea	(C <sub>4</sub> H <sub>8</sub> NO)CH <sub>2</sub> NHCSNH <sub>2</sub>	CY	100+	4	—	
n-Butyl thiourea	CH <sub>3</sub> (CH <sub>2</sub> ) <sub>3</sub> NHCSNH <sub>2</sub>	CY	200+	7	—	
Allyl thiourea	CH <sub>2</sub> CHCH <sub>2</sub> NHCSNH <sub>2</sub>	DP	300+	4	200	10
Isopropyl thiourea	(CH <sub>3</sub> ) <sub>2</sub> CH NHCSNH <sub>2</sub>	K	300+	4	500	8
Methyl thiourea	CH <sub>3</sub> NHCSNH <sub>2</sub>	EK	500+	12	±50	4
n-Lauryl thiourea	CH <sub>3</sub> (CH <sub>2</sub> ) <sub>11</sub> NHCSNH <sub>2</sub>	DP	1000+	3	1000	3
<b>C. N-Aryl Thioureas</b>						
2-Chlorophenyl thiourea	ClC <sub>6</sub> H <sub>4</sub> NHCSNH <sub>2</sub>	M	4.6 ± 0.4	24	—	
o-Tolyl thiourea	CH <sub>3</sub> C <sub>6</sub> H <sub>4</sub> NHCSNH <sub>2</sub>	DP	5.2 ± 0.7	27	1.5	12
2-Methyl,3-chlorophenyl thiourea	CH <sub>3</sub> (Cl)C <sub>6</sub> H <sub>3</sub> NHCSNH <sub>2</sub>	DP	6.3 ± 1.1	17	5	9
1-Naphthyl thiourea (ANTU)	C <sub>10</sub> H <sub>7</sub> NHCSNH <sub>2</sub>	DP	6.9 ± 0.5	50	2.5 ± 0.5	51
o-Biphenyl thiourea (with 10% biphenyl)	C <sub>6</sub> H <sub>5</sub> C <sub>6</sub> H <sub>4</sub> NHCSNH <sub>2</sub>	DP	8.5 ± 0.8	17	10.0 ± 0.9	29
Phenyl thiourea	C <sub>6</sub> H <sub>5</sub> NHCSNH <sub>2</sub>	EI	8.6 ± 0.6	30	3.1 ± 0.7	18
4-Chlorophenyl thiourea	ClC <sub>6</sub> H <sub>4</sub> NHCSNH <sub>2</sub>	M	15	16	—	
3-Chlorophenyl thiourea	ClC <sub>6</sub> H <sub>4</sub> NHCSNH <sub>2</sub>	M	25	12	—	
4-Bromophenyl thiourea	BrC <sub>6</sub> H <sub>4</sub> NHCSNH <sub>2</sub>	CY	50	8	—	
2-Naphthyl thiourea	C <sub>10</sub> H <sub>7</sub> NHCSNH <sub>2</sub>	DP	50	7	50?	15
4-Methoxyphenyl thiourea (crude)	CH <sub>3</sub> OC <sub>6</sub> H <sub>4</sub> NHCSNH <sub>2</sub>	B	75	12	—	
Xenyl thiourea	C <sub>6</sub> H <sub>4</sub> C <sub>6</sub> H <sub>4</sub> NHCSNH <sub>2</sub>	DP	100?	22	100?	9
4,4'-Biphenylene bis(thiourea)	NH <sub>2</sub> CSNH C <sub>6</sub> H <sub>4</sub> C <sub>6</sub> H <sub>4</sub> NHCSNH <sub>2</sub>	DP	200	12	±250	6
2,5-Dichlorophenyl thiourea	Cl <sub>2</sub> C <sub>6</sub> H <sub>3</sub> NHCSNH <sub>2</sub>	DP	300	9	325	6
2-Methoxyphenyl thiourea	CH <sub>3</sub> OC <sub>6</sub> H <sub>4</sub> NHCSNH <sub>2</sub>	B	300+	7	—	
Tetrahydro-2-naphthyl thiourea	C <sub>10</sub> H <sub>11</sub> NHCSNH <sub>2</sub>	DP	—		750?	6
<b>D. Other Singly N-Substituted Thioureas</b>						
Dithiobiuret	NH <sub>2</sub> CS NHCSNH <sub>2</sub>	CY	15	15	—5	10
Benzoylguanidyl thiourea	C <sub>6</sub> H <sub>5</sub> C(=O)NHC(=NH) NHCSNH <sub>2</sub>	CY	100+	4	—	
Phenylthioureylene thiourea	C <sub>6</sub> H <sub>5</sub> NHCSNH NHCSNH <sub>2</sub>	CY	100+	4	—	
Phenyl dithiobiuret	C <sub>6</sub> H <sub>5</sub> NHCS NHCSNH <sub>2</sub>	DP	500+	5	250	6

\* B = Dr A. F. Blakeslee, CY = American Cyanamid Company, DP = E. I. du Pont de Nemours and Company, EI = Eimer & Amend, EK = Eastman Kodak Company, K = Dr J. C. Krantz, Jr, M = Merck & Company, Inc

exhibited high toxicity. The same is presumably true of the guanidines, in which the doubly bound sulfur of thiourea has been replaced by an imido group. On the other hand the thioamide classification, in which one complete amino group of the thiourea structure is missing, provided three compounds which killed wild

TABLE 2

Symmetrically disubstituted thiourea derivatives  
(All contain thioureyne grouping:—NHCSNH—)

NAME	FORMULA	SOURCE*	WILD NORWAY RATS		DOMESTIC RATS	
			LD 50	No. used	LD 50	No. used
			mgm / kgm.		mgm / kgm.	
<b>A. N,N'-Dialkyl Thioureas</b>						
Di-n-butyl thiourea	$\text{CH}_3(\text{CH}_2)_3 \text{NHCSNH} (\text{CH}_2)_3 \text{CH}_3$	DP	350	8	1500+	6
Di-isopropyl thiourea	$(\text{CH}_3)_2\text{CH NHCSNH CH}(\text{CH}_3)_2$	DP	500	6	450	14
Di-allyl thiourea	$\text{CH}_2 \text{CHCH}_2 \text{NHCSNH CH}_2 \text{CHCH}_2$	CY	500+	4	—	
Di-cyclohexyl thiourea	$\text{C}_6\text{H}_{11} \text{NHCSNH} \text{C}_6\text{H}_{11}$	DP	500+	4	1500+	6
Di-2-methylcyclohexyl thiourea	$\text{CH}_3\text{C}_6\text{H}_{10} \text{NHCSNH} \text{C}_6\text{H}_{10} \text{CH}_3$	DP	500+	4	1500+	6
Polyhexamethylene thiourea	$(\text{NHCSNH} \cdot (\text{CH}_2)_6)_x$	DP	500+	4	1500+	6
Di-isobutyl thiourea	$\text{CH}_3\text{CH}_2\text{CH}(\text{CH}_3) \text{NHCSNH} \text{CH}(\text{CH}_3)\text{CH}_2\text{CH}_3$	DP	800	7	1000+	6
Di-octadecyl thiourea	$\text{CH}_3(\text{CH}_2)_{17} \text{NHCSNH} (\text{CH}_2)_{17}\text{CH}_3$	DP	—		500+	4
Di-lauryl thiourea	$\text{CH}_3(\text{CH}_2)_{11} \text{NHCSNH} (\text{CH}_2)_{11}\text{CH}_3$	DP	—		1500+	4
Allyl dodecyl thiourea	$\text{CH}_2 \text{CHCH}_2 \text{NHCSNH} (\text{CH}_2)_{11}\text{CH}_3$	DP	—		1500+	6
<b>B. N-Alkyl, N'-Aryl Thioureas</b>						
2-Hydroxyethyl 4-ethoxyphenyl thiourea	$\text{CH}_3\text{CH}_2\text{OC}_6\text{H}_4 \text{NHCSNH} \text{CH}_2\text{CH}_2\text{OH}$	B	100+	4	—	
p-Phenylene bis(allyl thiourea)	$\text{CH}_2 \text{CHCH}_2 \text{NHCSNH} \text{C}_6\text{H}_4 \text{NHCSNH} \text{CH}_2\text{CHCH}_2$	B	100+	4	—	
n-Butyl phenyl thiourea	$\text{CH}_3(\text{CH}_2)_3 \text{NHCSNH} \text{C}_6\text{H}_5$	CY, B	150	12	—	
Lauryl phenyl thiourea	$\text{CH}_3(\text{CH}_2)_{11} \text{NHCSNH} \text{C}_6\text{H}_5$	CY	300+	3	—	
Allyl phenyl thiourea	$\text{CH}_2 \text{CHCH}_2 \text{NHCSNH} \text{C}_6\text{H}_5$	DP	750	8	1200?	6
Allyl p-tolyl thiourea	$\text{CH}_2 \text{CHCH}_2 \text{NHCSNH} \text{C}_6\text{H}_4 \text{CH}_3$	DP	—		900?	9
2-Hydroxyethyl phenyl thiourea	$\text{HOCH}_2\text{CH}_2 \text{NHCSNH} \text{C}_6\text{H}_5$	DP	—		1500+	4
Allyl 4-Nitrophenyl thiourea	$\text{CH}_2 \text{CHCH}_2 \text{NHCSNH} \text{C}_6\text{H}_4 \text{NO}_2$	DP	—		1500+	4
<b>C. N, N'-Diaryl Thioureas</b>						
Phenyl o-tolyl thiourea	$\text{C}_6\text{H}_5 \text{NHCSNH} \text{C}_6\text{H}_4 \text{CH}_3$	B	100+	4	—	
Di-m-tolyl thiourea	$\text{CH}_3\text{C}_6\text{H}_4 \text{NHCSNH} \text{C}_6\text{H}_4 \text{CH}_3$	B	100+	4	—	
Phenyl 1-naphthyl thiourea	$\text{C}_6\text{H}_5 \text{NHCSNH} \text{C}_{10}\text{H}_7$	CY	100+	4	—	
4-Ethoxyphenyl 4-(2-dimethyl aminoethoxy phenyl) thiourea	$\text{CH}_3\text{CH}_2\text{OC}_6\text{H}_4 \text{NHCSNH} \text{C}_6\text{H}_4 \text{H}_2\text{N}(\text{CH}_3)_2$	B	100+	6	—	
Hydrochloride of preceding compound	$\text{CH}_3\text{CH}_2\text{OC}_6\text{H}_4 \text{NHCSNH} \text{C}_6\text{H}_4 \text{H}_2\text{N}(\text{CH}_3)_2 \text{HCl}$	B	100+	4	—	
p-Phenylene bis(phenyl thiourea)	$\text{C}_6\text{H}_5 \text{NHCSNH} \text{C}_6\text{H}_4 \text{NHCSNH} \text{C}_6\text{H}_5$	B	100+	4	—	
Di-2-chlorophenyl thiourea	$\text{ClC}_6\text{H}_4 \text{NHCSNH} \text{C}_6\text{H}_4 \text{Cl}$	M	200+	4	—	
Di-3-chlorophenyl thiourea	$\text{ClC}_6\text{H}_4 \text{NHCSNH} \text{C}_6\text{H}_4 \text{Cl}$	M	200+	4	—	
Di-4-chlorophenyl thiourea	$\text{ClC}_6\text{H}_4 \text{NHCSNH} \text{C}_6\text{H}_4 \text{Cl}$	M	200+	4	—	
Di-(2-methoxy, 5-methylphenyl) thiourea	$\text{CH}_3\text{O}(\text{CH}_2)_2\text{C}_6\text{H}_3 \text{NHCSNH} \text{C}_6\text{H}_3 \text{OCH}_3$	B	500+?	12	—	
Di-1-naphthyl thiourea	$\text{C}_{10}\text{H}_7 \text{NHCSNH} \text{C}_{10}\text{H}_7$	DP	1500+	3	1500+	9
Di-2-naphthyl thiourea	$\text{C}_{10}\text{H}_7 \text{NHCSNH} \text{C}_{10}\text{H}_7$	DP	2000+	4	2000+	10
Di-o-tolyl thiourea	$\text{CH}_3\text{C}_6\text{H}_4 \text{NHCSNH} \text{C}_6\text{H}_4 \text{CH}_3$	DP, B	2000+	7	2000+	5
Thiocarbamide	$\text{C}_6\text{H}_5 \text{NHCSNH} \text{C}_6\text{H}_5$	DP, EK	2000+	7	1500+	6
Di-4-fluorophenyl thiourea	$\text{FC}_6\text{H}_4 \text{NHCSNH} \text{C}_6\text{H}_4 \text{F}$	DP	—		1500+	6
Di-3-trifluoromethylphenyl thiourea	$\text{F}_3\text{CC}_6\text{H}_4 \text{NHCSNH} \text{C}_6\text{H}_4 \text{CF}_3$	DP	—		1500+	5

TABLE 2—Continued

NAME	FORMULA	SOURCE*	WILD NORWAY RATS		DOMESTIC RATS	
			LD 50	No used	LD 50	No used
			mgm / kgm		mgm / kgm	
<i>D Compounds with Thioureylene Grouping as Part of a Ring</i>						
2 Imidazolidinethione (Ethylene thiourea)	$\text{CH}_2 \text{ NHCSNH } \text{CH}_2$	CY	100+	3	—	
5-Chloro-2(3) benzimidazolethione	$\text{ClC}_6\text{H}_4 \text{ NHCSNH}$	M	150	7	—	
2-Thiouracil	$\text{CH } \text{CH } \text{NHCSNH } \text{CO}$	L	1000+	24	2000+	4
<i>E Other N, N'-Disubstituted Thioureas</i>						
o-Tolyl thiosemicarbazide	$\text{CH}_3\text{C}_6\text{H}_4 \text{ NHCSNH } \text{NH}_2(?)$	CY	200	11	—	
D: 2 pyridyl thiourea	$(\text{C}_5\text{H}_4\text{N}) \text{ NHCSNH } (\text{C}_5\text{H}_4\text{N})$	CY	200+	8	—	
D: pbenyl thiocarbazine	$\text{C}_6\text{H}_5\text{NH } \text{NHCSNH } \text{NHC}_6\text{H}_5$	DP	1500	4	1500+	4

\* B = Dr A F Blakeslee, CY = American Cyanamid Company, DP = E I du Pont de Nemours and Company, EK = Eastman Kodak Company, L = Lederle Laboratories, Inc, M = Merck and Company, Inc

TABLE 3

Other thiourea derivatives

(All contain thiourea nucleus:  $\text{=NCSN=}$ )

NAME	FORMULA	SOURCE*	WILD NORWAY RATS		DOMESTIC RATS		
			LD 50	No used	LD 50	No used	
			mgm / kgm		mgm / kgm		
<i>A Divalent Substituent on One Nitrogen</i>							
Ethylidene thiourea	$\text{CH}_3\text{CH NCSNH}_2$	CC	100+	4	—		
<i>B Two Substituents on Same Nitrogen N, N-Di benzyl thiourea</i>							
	$(\text{C}_6\text{H}_5\text{CH}_2)_2\text{NCSNH}_2$	CY	100+	4	—		
<i>C N, N', N''-Trisubstituted Thioureas</i>							
D: ethyl cyclohexyl thiourea	$(\text{CH}_3\text{CH}_2)_2\text{NCSNHC}_6\text{H}_{11}$	V	100+	4	—		
D: ethyl phenyl thiourea	$(\text{CH}_3\text{CH}_2)_2\text{NCSNHC}_6\text{H}_5$	V	100+	3	—		
<i>D N, N, N', N''-Tetrasubstituted Thioureas</i>							
3 (Dimethylthiocarbamyl) 2(3)-benzothiazolethione	$\text{C}_6\text{H}_5\text{SC( S)NC( S)N(CH}_3)_2$	V	100+	4	—		
3-(Diethylthiocarbamyl) 2(3)-benzothiazolethione	$\text{C}_6\text{H}_5\text{SC( S)NC( S)N(CH}_2\text{CH}_3)_2$	V	100+	4	—		
3 (Diethylthiocarbamyl), 6-methyl 2(3) benzothiazolethione	$\text{CH}_3\text{C}_6\text{H}_4\text{SC( S)NC( S)N(CH}_2\text{CH}_3)_2$	V	100+	4	—		

\* CC = Carbide and Carbon Chemicals Corporation, CY = American Cyanamid Company, V = R T Vanderbilt Company

Norways in doses less than 100 mgm /kgm., namely phenylthioacetamorpholide, 3-cyclohexene thiocarboxamide and 1-naphthyl thioacetamide

Two thiazolines likewise proved to have high toxicity: they were 2-mercapto-4-ethyl-thiazoline and 2-mercapto-4,4-dimethyl-thiazoline.

Of the 15 miscellaneous compounds listed in table 5, two of those tested on

**TABLE 4**  
*Compounds structurally related to Thiourea*

	SOURCE*	WILD NORWAY RATS		DOMESTIC RATS	
		LD 50	No. used	LD 50	No. used
		mgm./kgm.		mgm./kgm.	
<b>A. Thiopseudoureas (Isothioureas)</b> (All contain $\text{=NC(=S)-N-}$ grouping)					
S-(2-Benzothiazolylmethyl)-N,N-diethyl thiopseudourea hydrochloride	$\text{C}_6\text{H}_5\text{SC(=NH)N(CH}_2\text{CH}_3)_2 \cdot \text{HCl}$	CY	100+	4	—
Benzyl thiopseudourea hydrochloride	$\text{C}_6\text{H}_5\text{CH}_2\text{SC(=NH)NH}_2 \cdot \text{HCl}$	DP	180	12	500?
4-Nitrobenzyl thiopseudourea hydrochloride	$\text{NO}_2\text{C}_6\text{H}_4\text{CH}_2\text{SC(=NH)NH}_2 \cdot \text{HCl}$	DP	300	8	500?
Methyl thiopseudourea sulfate	$\text{CH}_3\text{SC(=NH)NH}_2 \cdot \text{H}_2\text{SO}_4$	DP	800	7	900+
S-Methyl,N,N'-diphenyl thiopseudourea	$\text{CH}_3\text{SC(=NC}_6\text{H}_5)_2\text{NEC}_6\text{H}_5$	DP	1500+	4	1500+
<b>B. Guanidines</b> (All contain $\text{=NC(=NH)NH-}$ grouping)					
1-(Dithiocyanomethylene)-3-cyano guanidine	$(\text{CNS})_2\text{C=NC(=NH)NHCN}$	CY	100+	4	—
Aminoguanidinesulfate	$\text{NH}_2\text{NHC(=NH)NH}_2 \cdot \text{H}_2\text{SO}_4$	DP	200+	4	—500
1,3-Diphenyl guanidine	$\text{C}_6\text{H}_5\text{NHC(=NH)NEC}_6\text{H}_5$	DP	—	—	500
1,3-Di-o-tolyl guanidine	$\text{CH}_3\text{C}_6\text{H}_4\text{NHC(=NH)NEC}_6\text{H}_4\text{CH}_3$	DP	—	—	500
Nitroguanidine	$\text{NH}_2\text{C(=NH)NHNO}_2$	DP	—	—	500+
Triphenyl guanidine	$\text{C}_6\text{H}_5\text{NHC(=NH)N(C}_6\text{H}_5)_2$	DP	—	—	750?
Carbamylguanidine sulfate	$\text{NH}_2\text{C(=NH)NHCONH}_2 \cdot \text{H}_2\text{SO}_4$	DP	—	—	500+
<b>C. Thioamides, etc.</b> (All contain $\text{=NC(=S)-}$ grouping)					
Phenylthioacetomorpholide	$\text{C}_6\text{H}_5\text{CH}_2\text{C(=S)NCH}_2\text{CH}_2\text{OCH}_2\text{CH}_2$	A	80	7	—
2-Cyclohexene thiocarboxamide	$\text{CH}_2\text{CH}_2\text{CH=CHCH}_2\text{CHCSNH}_2$	CC	50	12	—
1-Naphthyl thioacetamide	$\text{C}_{10}\text{H}_7\text{CH}_2\text{CSNH}_2$	CY	60	14	—
3,3'-Thio-bis(thiopropionamide)	$(\text{NH}_2\text{CSCCH}_2\text{CH}_2)_2\text{S}$	CC	100+	4	—
Thioformamide (Tech)	$\text{HCSNH}_2$	M	100+	4	—
N,N'-Diphenylguanyl thiocarboxamide	$\text{C}_6\text{H}_5\text{NH(C}_6\text{H}_5)_2\text{CCSNH}_2$	CY	100+	4	—
4-Bromo-thioformanilide	$\text{BrC}_6\text{H}_4\text{NHC(=S)H}$	CY	100+	3	—
4-Chloro-thiobenzamide	$\text{ClC}_6\text{H}_4\text{CSNH}_2$	CY	100+	4	—
Phenylthiocarbamate	$\text{C}_6\text{H}_5\text{OC(=S)NH}_2$	CY	100+	3	—
Thioacetanilide	$\text{C}_6\text{H}_5\text{NHC(=S)CH}_3$	CY	100+	4	—
Dithioacetamide	$\text{NH}_2\text{C(=S)CSNH}_2$	EK	200+	16	—
Thioacetamide	$\text{CH}_3\text{CSNH}_2$	EK	500+	8	—
<b>D. Thiazoles, etc.</b> (All contain $\text{—N=C(=S)—}$ grouping except one isothiazole with $\text{—SN=C=}$ )					
2-Mercapto-4-ethyl-thiazoline	$\text{CH}_3\text{CH}_2\text{CCHCH}_2\text{SC(SH):N}$	CY	35	16	—
2-Mercapto-4,4-dimethyl-thiazoline	$(\text{CH}_3)_2\text{CCHCH}_2\text{SC(SH):N}$	CY	40	8	—
2-Mercapto-5,5-dimethyl-thiazoline	$(\text{CH}_3)_2\text{CSC(SH):NCH}_3$	CY	100+	4	—
2-Mercapto-benzothiazole	$\text{C}_6\text{H}_5\text{SC(SH):N}$	CY, DP	100+	3	500+
2-Mercapto-5,6-dihydro-1,3,4-thiazine	$\text{CH}_3\text{CH}_2\text{CH}_2\text{SC(SH):N}$	CY	100+	4	—
2-Mercapto-thiazoline	$\text{CH}_2\text{CH}_2\text{SC(SH):N}$	DP	300	8	500+
4-Methyl-2-amino-benzothiazole hydrochloride	$\text{CH}_3\text{C}_6\text{H}_4\text{SC(NH}_2)_2\text{N HCl}$	DP	—	—	500
1,9-Isothiazole anthrone-2-carboxylic acid	$\text{O.CC}_6\text{H}_4\text{C=NSC}_6\text{H}_4\text{COOH}$	DP	500+	4	1500?
2-Amino-5,6,7,8-tetrahydro-naphthiazole hydrochloride	$\text{C}_{10}\text{H}_9\text{SC(NH}_2)_2\text{N HCl}$	DP	—	—	600?
2-(4-Nitrobenzoylamino)-6-nitro-benzothiazole	$\text{O}_2\text{NC}_6\text{H}_4\text{SC(NHCOC}_6\text{H}_4\text{NO}_2)_2\text{N}$	DP	—	—	1500
2-Mercapto-4-methyl-5-chloro-benzothiazole	$(\text{CH}_3)(\text{Cl})\text{C}_6\text{H}_3\text{SC(SH):N}$	DP	—	—	1500+

\* A = Dr. E. B. Astwood, CC = Carbide and Carbon Chemicals Corporation, CY = American Cyanamid Company, DP = E. I. du Pont de Nemours and Company, EK = Eastman Kodak Company, M = Merck and Company, Inc.

wild rats were toxic (cyclohexane sulfonamide and 4-dimethyl-amino-aniline hydrochloride) while one (coumarin-3-carboxylic acid) had a relatively high toxicity to domestic rats but was less toxic to wild Norways.

Included in table 6 are 6 dithiocarbamates, 11 amides and similar substances, 4 hydrazines (one other hydrazine is included in table 5), 30 amines, 6 nitriles (one other nitrile is to be found in table 5), and 13 miscellaneous compounds. The preliminary assays of these substances indicated low toxicity to domestic rats and hence none was assayed with wild Norway rats. On the basis of these very limited assays, their LD50's appear to be at least 500 mgm./kgm.

TABLE 5  
Miscellaneous

NAME	FORMULA	SOURCE*	WILD NORWAY RATS		DOMESTIC RATS	
			LD 50	No. used	LD 50	No. used
			mgm./kgm.		mgm./kgm.	
Cyclohexane sulfonamide	$\text{CH}_2(\text{CH}_2)_4\text{CHSO}_2\text{NH}_2$	DP	80	8	100?	7
4-Dimethylamino-aniline hydrochloride	$(\text{CH}_3)_2\text{NC}_6\text{H}_4\text{NH}_2\cdot\text{HCl}$	H	-90	4	—	—
Tetrahydro-2-naphthylamine hydrochloride	$\text{C}_{10}\text{H}_{11}\text{NH}_2\cdot\text{HCl}$	?	100	8	—	—
Dithioammeline	$\text{HSC:NC}(\text{NH}_2):\text{NC}(\text{SH}):N$	CY	100+	4	—	—
Thioammeline	$\text{H}_2\text{NC:NC:N}(\text{SH})(\text{NH}_2)\text{C:N}$	CY	100+	4	—	—
Phenyl arsonic acid	$\text{C}_6\text{H}_5\text{AsO}(\text{OH})_2$	DP	150	8	150?	7
Coumarin-3-carboxylic acid	$\text{C}_9\text{H}_6\text{OC}(\text{O})\text{C}(\text{COOH})\text{:CH}$	DP	200	12	20?	7
Aniline	$\text{C}_6\text{H}_5\text{NH}_2$	DP	—	—	-250	7
Isopertythiocyanic acid	$\text{NHC}(\text{S})\text{SSC:NH}$	CY	200+	4	—	—
1-Naphthyl amine	$\text{C}_{10}\text{H}_7\text{NH}_2$	DP	300	24	—	—
N-Ethylaniline	$\text{CH}_3\text{CH}_2\text{NHC}_6\text{H}_5$	DP	—	—	300?	7
Phenylacetone nitrile	$\text{C}_6\text{H}_5\text{CH}_2\text{CN}$	DP	—	—	-350	7
m-Toluidine	$\text{CH}_3\text{C}_6\text{H}_4\text{NH}_2$	DP	—	—	450?	7
Xenyl hydrazine	$\text{C}_8\text{H}_9\text{C}_6\text{H}_4\text{NHNH}_2$	DP	500+	4	1500+	4
Dicyanamide	$\text{NC:NH-CN}$	DP	500+	4	2000	4

\* CY = American Cyanamid Company, DP = E. I. du Pont de Nemours and Company, H = Dr. Leslie Hellerman, ? = source unverifiable

DISCUSSION. A study of the tabulated data shows that 19 compounds had LD50's below 100 mgm./kgm. for wild Norway rats, all the rest proving less toxic. In 12 of these the full  $-\text{NHCSNH}_2$  grouping occurred; that is, they differed from thiourea in the replacement of a single nitrogen-linked hydrogen atom by a substituent radical. In 5 others (3 thioamides, 2 thiazolines) the thiourea configuration was less complete in that one complete amino group had been lost, leaving only a  $-\text{CSNH}_2$  or a (potential)  $-\text{CSNH}-$  linkage. The last 2 compounds were unrelated to thiourea, being respectively a sulfonamide and the salt of an aniline derivative.

In general the results of the assays on domestic rats paralleled those for the

TABLE 6

*Other compounds\**

Found to have an LD50 of 500 mgm./kgm. or higher, on the basis of limited assays made only with domestic rats. The number of rats used in each case follows the name, in parentheses.

*A. Dithiocarbamates*

- Sodium dithiocarbamate (9)
- Zinc dimethyl dithiocarbamate (2)
- bis(Dimethylthiocarbamyl) monosulfide (2)
- bis(Dimethylthiocarbamyl) disulfide (2)
- bis(1-Piperidylthiocarbamyl) tetrasulfide (2)
- Piperidinium 1-piperidyl dithiocarbamate (2)

*B. Amides, etc.*

- Adipamide (2)
- Caprolactam (2)
- Trichloroacetamide (2)
- Carbanilide (2)
- N-1-naphthyl ethyl urethane (2)
- Ammonium sulfamate (2)
- N,N-Dimethyl-p-toluene sulfonamide (2)
- Barium diphenylamine sulfamate (2)
- N-Acetyl-2-amino-phenol (4)
- N,N'-Dimethyl carbanilide (4)
- p-Phenetyl urea (Dulcin) (4)

*C. Hydrazines*

- Hydrazine sulfate (2)
- 4-Bromophenyl hydrazine hydrochloride (7)
- Xenylene dihydrazine dihydrochloride (13)
- Phenyl hydrazine-p-sulfonic acid (4)

*D. Aliphatic amines*

- Ethylenediamine dihydrochloride (2)
- 1,6-Diamino-hexane hydrochloride (2)
- 1,10-Diamino-decane (2)
- Piperczinc hexahydrate (2)
- N,N'-Diphenylpiperazine hydrochloride (4)
- Laurylamine thiocyanate (4)

*E. Primary Aromatic Amines*

- Xenyl amine (2)
- Benzidine (2)
- 2-Amino-resorcinol hydrochloride (2)
- Arsanilic acid (2)
- p-Phenylene diamine (4)
- o-Toluidine (7)
- 4-Aminophenol (4)
- 3-Nitroaniline (4)
- 4-Nitroaniline (4)
- 4-Nitroaniline-2-sulfonic acid (4)

*F. Secondary Aromatic Amines*

- N,N'-Diphenyl ethylene diamine (2)
- 4-(Benzylamino)phenol (4)

TABLE 6—Concluded

*G. Tertiary Aromatic Amines*

- p-bis(Dimethylamino) benzene (2)
- p-Dimethylaminophenol oxalate (2)
- N,N'-Disalicylidene ethylene diamine (2)
- N,N-Dimethyl-1-naphthyl amine (7)
- N,N-Dibenzyl-4-amino-phenol (4)
- 4-(Dimethylamino) benzaldehyde (4)
- 4,4'-Methylene bis(N,N-dimethyl aniline) (4)
- 4,4'-bis(Dimethylamino) benzohydrol (4)
- Auramine (4)
- 4-(Benzalamino)phenol (4)
- N,N-Dimethyl-4-bromo-aniline (4)
- N,N-Dimethyl-o-toluidine (4)

*H. Nitriles*

- Pimelonitrile (7)
- Adiponitrile (7)
- 7-Aminoheptanonitrile (7)
- Sebaconitrile (10)
- 5-Amino-capronitrile (6)
- Lauronitrile (4)

*I. Miscellaneous*

- 2-Nitro-2-methyl-1,3-propanediol (2)
- Resorcinol (2)
- 2,4-Dinitrophenol (7)
- Tribromophenol (2)
- 4-Chloro-m-cresol (2)
- 3-Nitrosalicylic acid (2)
- Methylpentachlorophenyl ether (2)
- Isoamyl pentachlorophenyl ether (2)
- Coumarin (4)
- Butadiene cyclic sulfone (2)
- Cinchophen (2)
- Carbazole (2)
- Cyanuric acid (16)

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wild rats. There were, however, 4 compounds to which domestic rats were more sensitive, namely coumarin-3-carboxylic acid, methyl thiourea, acetyl thiourea and thiourea itself. Thus there were 3 compounds containing a thioureido linkage which were more toxic to domestic than to wild Norway rats. The response of rats from different colonies, or rats maintained on different rations, to the parent compound thiourea has been shown (5) to vary markedly, and presumably the explanation for this, when found, will apply to the methyl and acetyl derivatives as well.

Our data indicate that thioureas containing a benzene ring and the full thioureido grouping are much more likely to exhibit high acute toxicity than any other class of thiourea derivatives here studied. Thus phenyl thiourea was highly toxic

(LD50 8.6 mgm./kgm.) as were its various derivatives where substitution had occurred in the ring (2-, 3- or 4-chloro-, 2-methyl-, 2-phenyl-, or 2,3-phenylene-, which is ANTU, etc.). Further substitution on the thiourea part of the molecule led to reduced toxicity (N, N'-diphenyl thiourea, usually called thiocarbamide, had an LD50 above 2000 mgm./kgm.) as did ring closure (5-chloro-2(3)-benzimidazolethione had an LD50 of 150 mgm./kgm., compared to 25 mgm./kgm. for 3-chloro-phenylthiourea; these compounds are identical except that the second ring has been closed in the former). It will be noted also that doubling of the phenyl thiourea molecule to form 4,4'-biphenylene bis(thiourea) led to reduced toxicity (LD50 300 mgm./kgm.) even though two complete thioureido groups remained.

The aromatic thiourea derivatives tested form a homogeneous group, not only because they include the large majority of toxic compounds found in this study, but also in their physiologic action. They apparently share the capacity of causing increased capillary permeability in the lungs, which results in the production of pulmonary edema accompanied by sometimes spectacular amounts of pleural effusion. All rats receiving lethal quantities of these substances died after several hours or a day and showed these effects at autopsy, as did also the domestic rats succumbing to thiourea and several aliphatic derivatives. The pathology of acute poisoning by ANTU, which can be taken as typical, has been studied by Latta (6).

These same effects were also found in wild rats after poisoning with the 3 toxic thioamides, as well as in domestic rats given N,N'-di-isopropyl thiourea. Pleural effusion may also result from the administration of other disubstituted thiouras as well, although not in sufficient amounts to cause death; we have therefore not observed it.

Dithiobiuret, which is a toxic singly N-substituted thiourea without a benzene ring, killed without evidence of lung involvement; after low doses the rats appeared paralyzed at 4 days and remained totally inactive until they died, 5 to 12 days after poisoning. Thiopseudoureas and guanidines are pressor agents; they have been investigated by Smirk and his coworkers (7, 8), and by Hueper and Ichniowski (9). The toxic thiazolines killed rapidly, causing death in convulsions within an hour. So far as we know their physiologic properties have not been investigated.

Attention should be drawn to the relation between this work and that of Astwood and his associates (10, 11) and of McGinty and his associates (12, 13, 14), who are interested in derivatives of thiourea for quite a different purpose. Where we are looking for thiouras of high acute toxicity, with the aim of poisoning rats more efficiently, they want substances with low toxicities which will be efficacious in the treatment of thyrotoxicosis. It is interesting that compounds of the same series will satisfy two such diverse purposes, and particularly that multiple substitution and ring closure, which produce compounds undesirable from our point of view, are just the changes necessary to enhance their value as antithyroid agents.



TABLE 6—Concluded

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# DIGITALIS-LIKE ACTION OF SOME NEW GLYCOSIDES AND ESTERS OF STROPHANTHIDIN

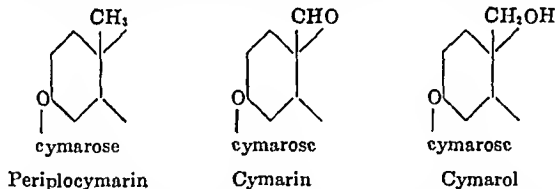
K. K. CHEN AND ROBERT C. ANDERSON

From the Lilly Research Laboratories, Eli Lilly and Company, Indianapolis 6

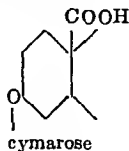
Received for publication May 5, 1947

During recent years Reichstein and his pupils (1) of Basel, Switzerland have carried out extensive investigations on the isolation and structural elucidation of various cardiac glycosides. At Columbia University, Elderfield and his co-workers (2) have synthesized compounds related to digitalis-like glycosides. The effort of this laboratory (3) has been directed to the pharmacological comparison of various cardiac substances. The data in the present communication, as shown in Table 1, cover 10 new glycosides generously supplied by Professor T. Reichstein, and 5 esters of strophanthidin generously supplied by Professor R. C. Elderfield.

Adonitoxin is a crystalline glycoside of *Adonis vernalis*, separated by Rosenmund and Reichstein (4); details of its structure have not as yet been published. Cymarol is a glycoside of *Strophanthus kombé*, isolated by Blome, Katz, and Reichstein (5). It differs from cymarin and periplocymarin by hydroxyl substitution on C<sub>1</sub>, as follows:



Sarmentosides A and B are the glycosides of *Strophanthus sarmentosus*, and cheirotoxin that of wallflower seeds, *Cheiranthus cheiri*, family *Cruciferae*; isolated, respectively, by Schmutz and Reichstein (6) and Schwarz, Katz, and Reichstein (7). The source of convallioside is the seeds of *Convallaria majalis*, and that of evonoside, the seeds of *Evonymus europaea* (8). Desgluco-hellebrin was obtained by partial hydrolysis of Karrer's diglycoside hellebrin, prepared from *Helleborus niger* (8). Methyl and potassium cymarylates are the reaction products of the following partial structure (9):



The 4 dialkylaminoacetyl strophanthidins and 3-chloroacetyl anhydrostrophanthidin were all made synthetically from the aglycone strophanthidin by Elderfield and his associates (10). Previously (11), a specimen of diethylaminoacetyl strophanthidin (synonymous with strophanthidin-3-diethylaminoacetate), m.p. 192.5° to 193.5°C., was examined. The present sample melted at a lower temperature, 169° to 170°C. This compound exists

## SUMMARY

On the basis of the assays reported above, the acute toxicity of thiourea to wild Norway rats is enhanced when a single aromatic radical is attached to one of the thiourea nitrogens. When there are 2 or more substituents, either on the same or both nitrogen atoms, the acute toxicity is lowered, as it also appears to be when substitution occurs on the sulfur atom, producing a thiopseudourea, or when the sulfur atom is replaced by an imido group to form a guanidine.

Pulmonary edema and pleural effusion resulted from acute poisoning with the N-substituted aromatic thioureas and with three of the thioamides, but were not produced consistently in lethal amounts by the other related compounds.

The results for domestic rats in general paralleled those obtained with wild Norways, differing markedly only in that the domestic rats exhibited a greater sensitivity to thiourea itself and to two aliphatic derivatives of low molecular weight.

*Acknowledgments.* We wish to express our gratitude to the following for supplying the chemicals used in our assays: Dr. E. B. Astwood, Tufts Medical School, Dr. A. F. Blakeslee, Smith College; Drs. E. K. Bolton, H. A. Lubs, J. E. Kirby, W. H. Tisdale, A. L. Flenner, Madison Hunt and R. S. Schreiber, E. I. du Pont de Nemours and Company, Mr. L. A. Brooks, R. T. Vanderbilt Company; Dr. Leslie Hellerman, Johns Hopkins Medical School; Dr. J. C. Krantz, Jr., University of Maryland Medical School; Dr. R. O. Roblin, Jr., American Cyanamid Company; Mr. C. A. Setterstrom, Carbide and Carbon Chemicals Corporation; Dr. H. N. Worthley, Merck and Company.

We should also like to express at this time our particular thanks to Dr. A. N. Richards, Chairman of CMR, OSRD, without whose continued encouragement and assistance this work could never have been done.

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the previous observation (14) that strophanthidin acid is many times less active than strophanthidin—a close similarity in each instance, both chemically and pharmacologically. The activity of cheirotoxin is very close to that of ouabain. While sarmentoside A is highly potent, sarmentoside B is devoid of any digitalis-like effect on the cat's heart. It is not improbable that the latter is in an alloform (6). Convallaside, a bioside, is weaker than convallotoxin, a monoside. The difference is similar to that between K-strophanthin- $\beta$  and cymarin. The activity of evonoside lies between that of cerberoside and that of thevetin.

TABLE 1  
*Results in cats*

COMPOUND	NUMBER OF CATS			DOSE RANGE TO KILL	MEAN (GEOMETRIC) LETHAL DOSE $\pm$ STANDARD ERROR
	Male	Female	Total		
				<i>mg. per kg.</i>	<i>mg. per kg.</i>
Adonitoxin.....	4	6	10	133.5- 263.0	191.3 $\pm$ 17.5
Cymarol.....	4	6	10	85.4- 162.6	99.4 $\pm$ 6.5
Sarmentoside A.....	3	6	9	93.8- 130.6	112.3 $\pm$ 5.4
Sarmentoside B.....	2	1	3	929.0-16572.0	failed to kill
Cheirotoxin.....	5	5	10	103.0- 135.7	118.5 $\pm$ 3.6
Convallaside.....	4	6	10	145.6- 263.4	215.0 $\pm$ 13.2
Desgluco-hellebrin.....	2	8	10	74.2- 114.2	86.1 $\pm$ 3.7
Evonoside.....	4	3	7	564.7- 1354.3	838.7 $\pm$ 119.5
Methyl Cymarylato.....		1	1	14100.0	failed to kill
Potassium Cymarylato.....		3	3	528.0-10720.0	3879.0
3-Dimethylaminoacetyl Stro- phanthidin.....	3	7	10	202.7- 290.8	266.3 $\pm$ 22.7
3-Diethylaminoacetyl Stro- phanthidin, m.p. 169-170°.....	3	7	10	201.1- 523.8	262.9 $\pm$ 40.5
3-Di-n-propylaminoacetyl Stro- phanthidin.....	3	7	10	172.7- 702.7	355.4 $\pm$ 47.9
3-Di-n-butylaminoacetyl Stro- phanthidin.....	5	5	10	440.3- 1288.8	704.5 $\pm$ 78.9
3-Chloroacetyl Anhydrostro- phanthidin.....		1	1	17120.0	failed to kill

Of the 4 alkylaminoacetyl strophanthidins, the dimethyl and diethyl are apparently equal in potency. Although the latter, the low melting form, shows a trend of being less active than the high melting form, as previously reported (11), the difference between the two is not highly significant because of the large standard error (see table 1). As the alkyl chain lengthens, the activity decreases—far greater in proportion than the molecular size. Thus, the di-n-propyl derivative, approximately 11.5% larger in molecular weight than the dimethyl derivative, is 33.5% less active. The disproportion is much greater if the di-n-butyl derivative is compared with the lower members of the series. The last compound in table 1 is inactive, which may be attributed to the absence of the hydroxyl group on C<sub>14</sub>. The result of this experiment is confirmatory of our previous observation that the hydroxyl group on C<sub>14</sub> is indispensable (3).

in two polymorphic forms, which are interconvertible by recrystallization. 3-Chloroacetyl anhydrostrophanthidin is a by-product in the preparation of chloroacetyl strophanthidin by the Küssner method (10).

With the exception of potassium cymarinate, all the products required various amounts of ethanol. As usual, special caution was exercised to limit the amount of alcohol to a minimum in order to avoid complications from an excessive quantity in animal experiments. Stock solutions of 1:1000 with 47.5% ethanol by volume were prepared with adonitoxin, cheirotoxin, cymarol, sarmentoside A, convallotoxin, desgluco-hellebrin, and evonoside; of 1:500 in 47.5% ethanol by volume, with sarmentoside B and 3-dimethylaminoacetyl strophanthidin; of 1:500 in 57.5% ethanol, with 3-diethylaminoacetyl and 3-di-n-propylaminoacetyl strophanthidins; of 1:1000 and 1:500 in 95% ethanol, respectively, with 3-di-n-butylaminoacetyl strophanthidin and 3-chloroacetyl anhydrostrophanthidin; and of 1:250 in 19% ethanol, with methyl cymarinate.

For cat experiments, dilutions of 1:200,000 in saline were made with stock solutions of cymarol and desgluco-hellebrin; of 1:100,000, with those of adonitoxin, sarmentoside A, cheirotoxin, and convallotoxin; of 1:50,000, with those of 3-dimethyl-, 3-diethyl-, 3-di-n-propyl-, and 3-di-n-butyl-, aminoacetyl strophanthidins; and of 1:25,000, with those of sarmentoside B, evonoside, and methyl cymarinate. An additional amount of ethanol was necessary to keep 2 of the above compounds in solution, namely, a dilution of 1:50,000 of 3-diethyl- and 3-di-n-butyl-, aminoacetyl strophanthidins had to have a total content of 2.85% and 3.8% ethanol by volume, respectively. Potassium cymarinate was completely soluble in saline solution—being used in a 0.186% solution.

The final dilutions of all the compounds were injected intravenously into cats until death occurred, in the same manner as previously reported (12). The rate of injection was 1 cc. per minute, except with sarmentoside B and 3-di-n-butylaminoacetyl strophanthidin it was 2 cc. per minute. 3-Chloroacetyl anhydrostrophanthidin was so insoluble in water that its stock solution had to be administered at the rate of 0.04 cc. per minute by means of a 3-way stopcock—a procedure adopted in this laboratory for insoluble substances (13). Of the 114 cats, the body weight varied from 1.704 to 2.935 kg., averaging 2.2258 kg. Groups of 10 cats each were employed for 9 substances; fewer animals, for the remaining 5 compounds—either because of limited quantity of material or lack of activity.

It is clear from the last column of table 1 that 6 of the natural glycosides have a high potency in cats. The activity of desgluco-hellebrin approaches that of convallotoxin (3), to date, the most potent of all glycosides investigated. Desgluco-hellebrin certainly can rank second. It remains to be seen whether the two substances have a close chemical resemblance. Cymarol is unquestionably more active in cats than cymarin and periploeymarin, results of which were previously reported (3)—showing that the presence of a carbinol group on  $C_{10}$  has a more favorable influence on cardiac activity than an aldehyde or a methyl group at the same position. The oxidation of this hydroxyl to a carboxyl group is, however, attended by an apparent loss of activity, since its methyl ester failed to kill a cat in the dose of 14.1 mg. per kg. (table 1). In large doses, the potassium salt was lethal to cats, but the changes in heart rate during the course of injection were not typical of digitalis action. Death could have been due to potassium ions. The correctness of this explanation is more probable if the results in frogs are considered. No systolic standstill occurred with doses as high as 0.6 mg. per g. injected into the lymph sac. It can be concluded, therefore, that the formation of an acid at  $C_{10}$  on the cymarin molecule results in a marked or complete loss of cardiac activity. Our contention is strengthened by

the previous observation (14) that strophanthidin is many times less active than strophanthidin—a close similarity in each instance, both chemically and pharmacologically. The activity of cheirotoxin is very close to that of ouabain. While sarmentoside A is highly potent, sarmentoside B is devoid of any digitalis-like effect on the cat's heart. It is not improbable that the latter is in an alloform (6). Convalloside, a bioside, is weaker than convallotoxin, a monoside. The difference is similar to that between K-strophanthin- $\beta$  and cymarins. The activity of evonoside lies between that of cerberoside and that of thevetin.

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3-Di-n-butylaminoacetyl Stro- phanthidin.....	5	5	10	440.3- 1288.8	704.5 $\pm$ 78.9
3-Chloroacetyl Anhydrostro- phanthidin.....		1	1	17120.0	failed to kill

Of the 4 alkylaminoacetyl strophanthidins, the dimethyl and diethyl are apparently equal in potency. Although the latter, the low melting form, shows a trend of being less active than the high melting form, as previously reported (11), the difference between the two is not highly significant because of the large standard error (see table 1). As the alkyl chain lengthens, the activity decreases—far greater in proportion than the molecular size. Thus, the di-n-propyl derivative, approximately 11.5% larger in molecular weight than the dimethyl derivative, is 33.5% less active. The disproportion is much greater if the di-n-butyl derivative is compared with the lower members of the series. The last compound in table 1 is inactive, which may be attributed to the absence of the hydroxyl group on C<sub>14</sub>. The result of this experiment is confirmatory of our previous observation that the hydroxyl group on C<sub>14</sub> is indispensable (3).

Six of the natural glycosides were further tested in frogs. In view of the fact that the sensitivity of frogs varies so much more than that of cats, simultaneous assays were carried out, a few at a time. Thus, in table 2, it can be noted that cymarol, cymarin, and adonitoxin were studied at the same time. The same is true with cheirotxin and cymarin; sarmentoside A and ouabain, and convallioside, desgluco-hellebrin, and cymarin.

When the median systolic doses in table 2 and the mean lethal doses in table 1, or in our earlier publication (3), are compared, it becomes clear that the order of activity is frequently reversed. For example, cymarol is more potent than cymarin in cats, but weaker than cymarin in frogs. A similar reversal exists between cymarol and cymarin, on the one hand, and adonitoxin, on the other. Cheirotxin is more than twice as active as cymarin in frogs, but definitely less active in cats. Sarmentoside A is also twice as active as ouabain in frogs, but

TABLE 2  
*Results in frogs*

GLYCOSIDE	NUMBER OF FROGS USED	MEDIAN SYSTOLIC DOSE $\pm$ STANDARD ERROR
		<i>mm Hg</i>
Cymarin .	30	0.810 $\pm$ 0.030
Cymarol	40	1.120 $\pm$ 0.120
Adonitoxin	35	0.621 $\pm$ 0.046
Cheirotxin .	35	0.410 $\pm$ 0.040
Cymarin	35	0.880 $\pm$ 0.070
Sarmentoside A	60	0.509 $\pm$ 0.560
Ouabain	30	1.141 $\pm$ 0.088
Convallioside	30	1.090 $\pm$ 0.090
Desgluco-hellebrin	35	0.290 $\pm$ 0.023
Cymarin	30	0.695 $\pm$ 0.055

almost equally active in cats. Such discrepancies have been previously observed between scillaren B and ouabain (15). A suggestion of agreement of results between cats and frogs occurs between convallioside and desgluco-hellebrin, on the one hand, and cymarin, on the other.

#### SUMMARY

1. The activity of 10 cardiac glycosides and 5 esters of strophanthidin has been determined in cats by intravenous injection: adonitoxin, cymarol, sarmentosides A and B, cheirotxin, convallioside, desgluco-hellebrin, evonoside, methyl and potassium cymarylates; 3-dimethyl-, 3-diethyl-, 3-di-*n*-propyl-, and 3-di-*n*-butyl-, aminoacetyl strophanthidins; and 3-chloroacetyl anhydrostrophanthidin.

2. The significance of certain structural changes has been pointed out

3. The potency of 6 glycosides (adonitoxin, cymarol, cheirotxin, sarmentoside

A, convallioside, and desgluco-hellebrin) has also been determined in frogs, and compared simultaneously with that of cymarin or ouabain. The order of activity of these substances is frequently reversed between cats and frogs.

*Note:* Dr. Maximilian Ehrenstein, George S. Cox Medical Research Institute, University of Pennsylvania, generously supplied us with specimens of strophanthidol and 3,19-diacetyl strophanthidol, which he and Johnson synthesized (16). Our pharmacologic results on these two compounds have a direct bearing on the present communication. The mean (geometric) lethal dose of strophanthidol in 10 cats is  $285.4 \pm 37.1$   $\mu\text{g.}$  per kg., being more potent than both strophanthidin and periplogenin. The order of activity, therefore, corresponds to that of cymarol on the one hand, and cymarin and periplocymarin on the other; again indicating on  $\text{C}_{10}$ , alcohol > aldehyde > methyl. 3,19-Diacetyl strophanthidol failed to kill cats in doses as large as 8930 and 15110  $\mu\text{g.}$  per kg. Similarly, it did not produce systolic arrest in frogs in doses varying from 259 to 478  $\mu\text{g.}$  per g., although their hearts ceased to beat. The disappearance of digitalis-like action in this case is unexpected in view of the increase in potency by acetylation of the OH group on  $\text{C}_3$  of strophanthidin.

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# STUDIES ON THE MECHANISM OF DEATH IN DOGS AFTER SYSTEMIC INTOXICATION BY THE INTRAVENOUS INJECTION OF METHYL-BIS( $\beta$ -CHLOROETHYL)AMINE OR TRIS( $\beta$ -CHLOROETHYL)AMINE.<sup>1</sup>

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In a recent background summary of the biological actions and therapeutic applications of the  $\beta$ -chloroethyl amines and sulfides (1), systemic pharmacology and pathology of methyl-bis( $\beta$ -chloroethyl)amine (officially designated as HN2) and tris( $\beta$ -chloroethyl)amine (officially designated as HN3) in man and experimental animals have been described. The most prominent systemic effects of these agents are upon the bloodforming organs with the production of leucopenia, and upon the mucosa of the gastrointestinal tract, where injury results in copious loss of fluid through vomiting and diarrhea which, with anorexia, lead to a marked reduction in body weight. Terminally, circulatory failure leads to coma and death appears to result from respiratory failure. Gross and micropathologic studies reveal extensive lesions in the intestinal tract, bone marrow and lymphatic tissue.

The present investigation was undertaken to evaluate the fluid loss and circulatory status in the dog, and to try to determine to what extent these changes could contribute to the death of the animal. Changes which might be the cause of death were sought in various plasma constituents, blood gases, properties of whole blood and red cells after withdrawal from the body, and in the body water compartments. Two general methods have been used in an attempt to evaluate the significance of fluid loss in intoxicated animals: 1) the replacement of fluid, electrolyte and protein by single or repeated subcutaneous or intravenous injections and 2) protection of the small intestine by occlusion of its blood supply during and for 15 minutes after the injection of the toxic agent, with the hope of preventing enteritis and fluid loss. It has been demonstrated that the intestine of the rat (HN2 and mustard) and rabbit (mustard) can be protected in this manner (2).

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<sup>3</sup> Much of the experimental work reported in this paper has been carried out with the assistance of Margaret Wing. Grateful acknowledgement is made to Dr. H. M. Zimmerman (Yale University), Dr. Irving Graef, Dr. Val Jager and Dr. Boris Krichesky for the pathologic observations, to Dr. L. James Talbot and Dr. Kenneth Cosgrove for performing the splenectomies, to Dr. Boris Krichesky for performing the intestinal clamping operations, to Elesá C. Addis and Mildred Bevelander for the blood counts, to Ann Lovell for some of the manometric analyses, and to Robert B. Golbey for assistance in carrying out the parenteral feeding experiments.

In the blood biochemical studies methyl-bis( $\beta$ -chloroethyl)amine (HN2) was administered to dogs intravenously in freshly prepared saline solutions of the hydrochloride in a dose of 1 mgm./kgm. Because of the general similarity between the known features of systemic intoxication produced by this compound and tris( $\beta$ -chloroethyl)amine (HN3), subsequent studies have been carried out with the latter compound, the hydrochloride of which was administered intravenously in a dose of 1 mgm./kgm.

**EXPERIMENTAL AND ANALYTICAL PROCEDURES.** Oxygen capacity and oxygen saturation of arterial blood, oxygen saturation of venous blood (jugular vein), and carbon dioxide capacity of arterial blood were determined by manometric analyses in the Van Slyke-Neill constant volume gas analysis apparatus (3). Carbon dioxide capacity ( $T_H$ ) was calculated at a  $CO_2$  tension of 40 mm. Hg according to Van Slyke and Sendroy (4).

Blood pH was determined by the Cambridge Electron Ray pH Meter, utilizing a McInnes electrode without exposure of the blood to air or oil.

Lactic acid concentration of plasma was determined by the method of Barker and Summerson (5), chlorido concentration of plasma by the method of Van Slyke (6), hemoglobin by the colorimetric method of Evelyn and Malloy (7) or calculated from the oxygen capacity (3), total plasma protein concentration by the micro-Kjeldahl method of Howe (8) or the copper sulfate method of Phillips *et al.* (9), and non-protein nitrogen concentration by the method of Koch and McMeekin (10). Extracellular fluid volume was taken as the volume of distribution of sodium thiocyanate (11) 60 minutes after the intravenous injection of known quantities of this substance and without correction for excretion, which was considered negligible. Sedimentation rate and hematocrit were determined by transferring a one cc. sample of well-mixed blood directly from the syringe into a Wintrobe hematocrit tube, which was placed in a water bath at 37°C. Readings of the sedimentation rate were made at 10 minute intervals for an hour. The tubes were then centrifuged for 40 minutes at high speed for the determination of the hematocrit. Osmotic fragility of red cells was determined by measuring the hemoglobin content of the supernatant fluid obtained from 0.1 cc. of whole blood plus 10 cc. of sodium chloride solutions ranging from 0.1 to 0.7 per cent, the unhemolyzed cells being first removed by centrifugation.

Plasma volume (PV) and total cell volume were determined by injection of known quantities of the dye T-1824 into the jugular vein (12) and subsequent withdrawal of four samples of blood at 15 minute intervals for the determination of plasma dye concentration (13, 14). In some experiments, after determining the plasma and cell volumes, from 100 to 150 cc. of human or heparinized pooled dog plasma, 8 per cent gelatin, or 6 per cent acacia in saline or in 20 per cent glucose were injected intravenously in an attempt to bring into circulation possibly sequestered or trapped cells. After waiting 20 minutes to one hour to insure thorough mixing of the injected fluid and colloid, a second intravenous injection of dye was made for a redetermination of plasma and total cell volumes.

Glomerular filtration rate was measured by the clearance of creatinine (15) and effective renal plasma flow by the clearance of p-aminohippuric acid (PAH) (16). Plasma samples were precipitated for creatinine and PAH determinations by the cadmium sulfate method of Fujita and Iwatake (17) and the creatinine concentration of the plasma filtrate and diluted urines was determined by the method of Folin and Wu (18). PAH was determined by the method of Smith, Finkelstein, Aliminoso, Crawford and Graber (16). In renal clearance experiments, ten per cent mannitol was infused intravenously at the rate of 1.5 cc./min. as a diuretic. Mean arterial blood pressure was recorded by femoral arterial puncture and a mercury manometer immediately before and after each clearance study.

**Surgical procedure.** Dogs weighing 5 to 7 kgm. were anesthetized by intravenous nembutal. The abdomen was opened by a midline incision in the epigastrium and the small intestine delivered from the wound. Rubber ties were passed around the small intestine at

the upper end of the jejunum and at the ileocecal junction. The superior mesenteric artery and its immediate branches were then located, cleared and clamped with rubber-sheathed hemostats. The rubber ties about the gut were drawn tight and the vessels were watched for pulsation. The presence of deep cyanosis of the intestine and the absence of pulsation in the vessels was considered adequate evidence of stasis. The toxic agent was then injected into the jugular vein. The clamps and ties about the gut were maintained in place for 15 minutes and then removed. The bowel was observed until the return of adequate circulation; then a small amount of sulfadiazine powder was left in the peritoneal cavity and the abdomen closed. The dogs were given 25 mgm. of demerol subcutaneously or intramuscularly during the first hour after the operation.

**RESULTS AND DISCUSSION.** *Toxicity.* A single intravenous injection of 1.0 mgm./kgm. of HN2·HCl or HN3·HCl in fasting dogs was found to be approximately an LD<sub>50</sub> for HN2 and LD<sub>76</sub> for HN3. It appears that HN3 is more toxic than HN2 in dogs by this route, as in rats, mice and rabbits (19). The mean death time after HN2 was 125 hours; after HN3, 76 hours (Table 3).

*Symptoms.* The symptoms of dogs intoxicated by a single intravenous injection of HN2 or HN3 are as follows: vomiting begins within a few hours after intoxication, increasing in severity and generally continuing through the second and third day. Vomiting, both early and late, may reflect an early neurogenic disturbance, or injury of the intestinal mucosa. Diarrhea, usually blood-stained or frankly hemorrhagic, is generally present on the second to fourth day (diarrhea was present at some time in 93 per cent of 105 dogs receiving an LD<sub>50</sub> of HN2 or HN3 intravenously). Intestinal bleeding is considered to be of capillary origin since larger bleeding points were not demonstrated.

Together with profuse vomiting and diarrhea, there is progressive loss of fluid, electrolyte and protein, as revealed by the following biochemical and fluid balance data:

*Extracellular fluid volume* decreased by 16 per cent on the fourth day of intoxication in one dog dying at 10 days after intoxication (HN2), by 22 per cent on the fifth day in a dog dying at eight days, and by 30 per cent on the fourth day in a dog dying two hours after the determination. Extracellular fluid decreased by 17 per cent on the fifth day in one surviving dog. This degree of reduction of extracellular fluid appears to be physiologically significant.

*Plasma volume* decreased by an average of 20 per cent (range 3 to 46) by the third day of intoxication (HN3), as observed in 19 dogs, 14 of which were splenectomized 10 days or more before intoxication to eliminate this organ as a possible site of sequestration of cells (Table 1). This is of the same order of magnitude of change as is observed in shock following skeletal trauma and hemorrhage (25 to 15 per cent, respectively) although it is less than the average figure of 40 per cent reported in cases of burn shock in man (21).

*Whole blood volume* decreased by an average of 15 per cent (range 1 to 36)

\* In four dogs receiving 10 daily intravenous injections of 0.1 mgm./kgm. of HN3·HCl (total dose = 1.0 mgm./kgm.), the symptoms characteristic of a single intravenous dose of 1.0 mgm./kgm. were absent, and the only evidence of intoxication was a moderate reduction in the total leucocyte count. In each instance, recovery of the count began before the fifth day.

TABLE 1

Mortality data, clinical observations, and plasma, blood and extracellular fluid volumes after various experimental procedures on dogs intoxicated by the intravenous injection of 1 mgm/kgm HN3 HCl (An ID<sub>50</sub>)

EXPERIMENTAL PROCEDURE	NO. OF DOGS	DIARRHEA*	VOMITING*	AVERAGE PER CENT GAIN OR LOSS AT 72 HOURS†					Filtrable fluid volume
				Body wt	Plasma volume	Blood volume	Total circulating proteins		
Unoperated, Intoxicated, No therapy	16	4/16— 7/16++ 1/16+++ 4/16++++	2/16— 4/16+ 7/16++ 3/16+++	-11 (-6 to -21)	-20 (+2 to -54)	-16 (0 to -36)	-16‡ (+11 to -36)	—	
Unoperated, Intoxicated, i.v. saline, glucose, plasma, gelatin or acetin	31	3/31— 1/31+ 13/31++ 9/31+++ 7/31++++ 1/31+++++	4/31— 16/31+ 13/31++ 3/31+++ 1/31++++ 1/31+++++	—	—	—	—	—	
Operated, 4 normal	2	2/2—	2/2—	+4.5 (0 to +9)	+7.1 (-3.5 to +17.5)	-0.5 (-11.5 to +10.5)	-3.8‡ (-5.6 to -2.1)	+7.5 (-4.5 to +17.5)	
Operated, Intoxicated, No therapy	6	5/6— 1/6+	6/6—	-0.6 (-6 to -16)	-5.1 (0 to -30)	-10 (+5 to -25)	-11§ (0 to -32)	-15 (-9 to -27)	
Operated, Intoxicated, 800 cc saline s.c./day	10	3/10— 6/10+ 3/10++	2/10— 6/10+ 1/10+++ 1/10++++	—	—	—	—	—	
Operated, Intoxicated, Amigen and glucose i.v.	4	4/4—	2/4— 1/4+ 1/4++	-8 (-14 to +10.5)	-14.4 (-30 to +4.5)	-3.9 (-11.5 to +1.6)	-13.8   (-29.5 to +7.7)	—	

\* Diarrhea and vomiting are graded from complete absence (—) to severe, copious discharge (+++).

† Values in parentheses indicate the highest and lowest values obtained.

‡ Plasma protein concentration determined by micro-Kjeldahl analysis (3).

§ Operation—occlusion of blood supply to small intestine during and for 15 minutes after intravenous injection of HN3.

|| Plasma protein concentration determined by copper sulfate specific gravity method (9).

(Table 1), considerably less than the 30 to 35 per cent reduction reported for man in shock from skeletal trauma, hemorrhage or burns (21).

It is difficult to visualize the relative importance of plasma loss as compared with whole blood loss, but it is our opinion that the decrease in whole blood volume alone is inadequate to cause death and other contributory factors must be sought, such as loss of protein and electrolytes.

*Plasma chloride concentration* fell in nearly all fatally intoxicated dogs (HN2), i.e., -1, -11, -15, -18, -19, -20, -29, -33 per cent of control values at the last determination. In 6 surviving dogs, chloride concentration did not change significantly.

*CO<sub>2</sub> capacity (T<sub>40</sub>)* increased in 4 of 4 intoxicated dogs (HN2). This increase presumably was a consequence of the predominance of loss of acid by vomiting over loss of base by diarrhea.

*Blood pH* in 6 dogs studied increased terminally in every case, the highest value observed being 7.72. Since in most cases respiration was not markedly depressed and pulmonary ventilation was not impaired, this increase in pH indicates an alkalosis arising mainly from loss of fixed acid. However, in some instances a terminal hyperventilation may have been a contributory factor.

*Lactic acid* was studied in 2 dogs only and did not seem to vary significantly during the course of intoxication. These observations, combined with the data on carbon dioxide capacity and pH, indicate that there is no significant accumulation of fixed acid.

*Total circulating protein* (total plasma protein concentration times plasma volume) decreased by an average of 21 per cent (range 1.6 to 36.5) in 16 of 19 dogs (HN3) (Table 1); in the absence of edema and proteinuria, the most likely explanation of this decrease is loss of protein in the diarrheic stool. Protein increased 10 per cent (range 6.7 to 14.0) in 3 of 19 dogs. The protein loss in man in burn shock, skeletal trauma, and hemorrhage may be of the order of 35 per cent or more (21).

*Total plasma protein concentration* in 9 fatally intoxicated dogs (HN2) had changed, in terminal observations, by -36, -8, +1, +28, +32, +38, +43, +85 and +134 per cent of the control value, although in some instances the degree of concentration was substantially greater one or two days before the terminal observation. In 3 surviving dogs, the change was -13, +7, and +14 per cent. An increase of 13 per cent was also observed in 14 of 19 dogs 72 hours after intoxication (HN3). In 5 of 19 dogs, the concentration decreased. The increased concentration in the majority of cases indicates that more water than protein is lost from the circulation. The loss of protein offsets the increase in protein concentration which otherwise would be expected from fluid loss. In man after hemorrhage and skeletal trauma, the reduction in plasma protein concentration is of the order of 20 per cent; in burn shock, 8 per cent (21). It is apparent that in HN3 intoxication relatively much more water than protein is lost than is the case in man in shock; it is therefore inferred that the fatal effects of HN3 intoxication cannot be attributed to protein loss *per se*.

*Non-protein nitrogen concentration* of plasma rose in 5 and fell in 2 fatally intoxicated dogs (HN2), and remained constant in one surviving dog. However, not much significance can be attached to this figure. Glomerular filtration in rabbits intoxicated by the intravenous administration of HN2-HCl is not impaired until circulatory collapse occurs (22) and azotemia when present is unquestionably of pre-renal origin.

*Total red cell volume* as calculated from the plasma volume and the hematocrit decreased in 13 of 19 dogs 72 hours after intoxication (HN3) by an average of 63 cc. of cells, but increased in 6 of 19 dogs by an average of 41 cc. The reduction exceeded 10 per cent in only 8 of 19 dogs, including 2 survivors. The decrease in total cell volume might be a result of hemorrhage through the intestinal wall or of *in vivo* sequestration or trapping of cells. Most of the dogs had bloody diarrhea when total red cell volume determinations were made. However, in none of the 8 dogs which showed a decrease in total red cell volume over 10 per cent had blood been present in the stool up to the time of examination.

*Total red cell volume after fluid and protein infusion.* The administration of fluids and colloids on the third day after intoxication has failed to bring into circulation any significant quantity of cells. In fact, in most instances total red cell volume actually decreased as a consequence of hemodilution following fluid administration. Acacia infusion resulted in an average increase of 16 per cent in total cell volume (range 2 to 30) in 4 of 7 dogs, and a decrease of 6 per cent (range 3 to 9) in 3 of 7. Human plasma produced an average decrease of 11 per cent (range 9 to 13) in 3, an increase of 3 per cent in 2, and no change in 1 of 6 dogs. Dog plasma infusion resulted in an average decrease of 13 per cent in 3 of 3 dogs. Gelatin produced a decrease of 16 per cent in 2 of 3 with an increase of 3 per cent in 1 of 3 dogs.

It is apparent that if sequestration of red cells occurs in HN3 intoxication, these cells cannot be restored to the circulation by temporary restoration of plasma volume with any of the media studied above. Failure to restore cells to the circulation is, however, not a convincing argument against sequestration, because similar infusions administered to normal dogs frequently result in a marked reduction in total circulating cell volume. Furthermore, the extent of such sequestration may be masked by a change in individual cell volume. However, we are inclined to believe that in HN3 and HN2 intoxication, extensive sequestration does not occur, because the actual loss of red cells is significant in only half of the animals studied.

*Total circulating protein after fluid and protein infusion.* A comparison of total circulating protein before and after injection of human plasma, relative to the amount of protein administered, revealed a loss of considerable protein one hour after infusion, averaging 14 per cent (range 7 to 22) in 6 of 6 dogs. An average of 5 per cent (range 0.4 to 12) disappeared after dog plasma in 3 of 3 dogs. In the case of gelatin, 3 dogs lost an average of 21 per cent (range 4 to 30). A discrepancy of the same magnitude between the expected and calculated total circulating protein after fluid injection was also observed in several

normal dogs and therefore the disappearance of protein in intoxicated animals cannot be attributed to increased capillary permeability.<sup>5</sup>

Splenectomized animals did not differ significantly from dogs with intact spleens with respect to alteration of plasma and total cell volume or circulating protein on the third day of intoxication, either before or after fluid or colloid infusion, thus eliminating this organ as a haven for sequestered red cells or protein

The hematocrit in 12 of 19 dogs intoxicated with HN3 increased an average of 17.5 per cent (range 1 to 38), and decreased in 4 of 19 dogs, in one instance by as much as 30 per cent. The hemoconcentration present in most dogs reflects a greater loss of fluid than cells from the circulatory system. This hemoconcentration need not necessarily be proportionate to the reduction in plasma volume in the presence of cell loss or change in cell size.

*Hemoglobin determinations* varied considerably. The results at their face value suggest a reduction of hemoglobin content in the terminal stages of intoxication. *Methemoglobin* did not appear in significant amounts in any of the dogs studied.

*Oxygen capacity* in 3 dogs (HN2) on the day of death increased by 11 (second day) and 19 per cent (fourth day), and fell by 16 per cent (second day). In one survivor there was no significant variation at any time during the first five days. Failure of oxygen capacity to increase as markedly as would be expected if hemoconcentration paralleled the increase in plasma protein concentration suggests the loss of hemoglobin or red cells.

*Changes in properties of blood withdrawn from intoxicated animals* include increased coagulation time, increased sedimentation rate, and increased tendency of cells to agglutinate into large, irregular masses in which individual cells are indistinguishable. No change in osmotic fragility of red cells was observed.

*Reduction of body weight* is associated with the excessive fluid and protein loss. All fatally intoxicated dogs lost an average of 3.8 per cent of their body weight per day (11.3 per cent by 72 hours, Table 1), which is more extensive than the mean loss in normal, fasting dogs allowed water *ad libitum* (ca. 1.57 per cent per day) (24, 25).

*Terminal weakness and coma* preceding death occur in the majority of dogs on the third to fifth day after intoxication by either HN2 or HN3. They are associated with low mean femoral arterial blood pressure, usually between 20 and 45 mm Hg, marked oxygen unsaturation of jugular blood (with presumably normal arterial oxygen saturation), reduction in body temperature, coldness of extremities, relaxation of the anal sphincter, and finally respiratory failure.

\* This is supported by the observation that the disappearance rate of dye T-1824, considered as a measure of the rate of escape of plasma albumin from the blood (23), did not increase by the third day of intoxication. However, a possibly significant increase in the disappearance rate after infusion of fluids on the third day of intoxication indicates an increased loss of albumin, which agrees with the observed decrease in the total circulating proteins one hour after fluid infusion.



The oxygen saturation of jugular blood was markedly depressed at one time or another in 4 dogs (HN2), but had returned to a normal or supernormal value in one lethally intoxicated dog on the day of death. Despite this single instance, it was grossly apparent that the jugular blood was with fair consistency excessively unsaturated during roughly the last two days of intoxication. The saturation of mixed venous blood in the rabbit was shown to decrease terminally. It is our opinion that these facts can best be explained on the basis of reduced blood flow rather than on the basis of increased oxygen utilization by the tissues.

Arterial oxygen saturation was studied in 7 unanesthetized rabbits (blood obtained by cardiac puncture) after receiving 3 mgm./kgm. HN2·HCl intravenously (a 2 LD<sub>50</sub>). Since in this species no excessive unsaturation was present, this measurement was omitted in the dog. The average arterial saturation on 24 normal rabbits was 91.4 per cent. In intoxicated animals prior to death, arterial saturation in those surviving one day was 78.2, 85.8, and 83.7 per cent; surviving three days, 98.7 per cent; surviving four days, 91.7 and 95.1 per cent; and surviving five days, 85.7 per cent. Four of seven of these values are definitely low but not sufficiently reduced to warrant the conclusion that there was significant pulmonary injury.

Excluding a few animals with severe pulmonary injury or infection as a complication, it is inferred that death is caused by anoxia of the respiratory centers as a consequence of peripheral circulatory failure precipitated chiefly by a reduction of blood volume attributable to loss of protein, electrolyte and water through vomiting and diarrhea, supplemented by loss of red cells through diarrhea and possibly unidentified channels. That undescribed pathologic changes may contribute to the fatal effect is indicated by the fact that some animals in which reduction of plasma and extracellular fluid volume by the third day after intoxication was not prominent died at approximately the same time after intoxication and with identical signs as did animals in which these changes were more marked. However, it must be pointed out that in the majority of cases these measurements were made routinely on the third day of intoxication when, with some variation, the animals were quite sick, and since such studies were not continued until death, it is possible that before death a further fluid, protein and cell depletion may have occurred of sufficient extent to produce circulatory failure. Death from vascular collapse cannot be ruled out in these animals.

*Renal clearance studies.* Page (26) has adduced evidence that a vasoconstrictor substance appears in the blood of animals in which shock has been induced by tourniquet, trauma, hemorrhage or burn. Corcoran, Taylor and Page (27) have reported that the renal blood flow is decreased and the filtration fraction increased in dogs suffering tourniquet shock. Phillips *et al* (28) have found essentially the same response in dogs after either progressive hemorrhage or muscle trauma. These renal changes are consistent with efferent arteriolar constriction and are independent of changes in the systemic blood pressure.<sup>6</sup> A

<sup>6</sup> Lauson, Bradley and Cournand (29) have found that a decrease in renal blood flow with no change in filtration fraction accompanies the fall in cardiac output in shock in man.

TABLE 2  
Renal clearances in fasting dogs intoxicated with HN3

DOG NO.	TIME AFTER INTOXICATION	MEAN ARTERIAL BLOOD PRESSURE	HEMATO- CRIT	FILTRATION RATE	EFFECTIVE RENAL PLASMA FLOW	EFFECTIVE RENAL BLOOD FLOW*	FILTRATION FRACTION†
	hours	mm. Hg	% cells	cc./m <sup>2</sup> /min.	cc./m <sup>2</sup> /min.	cc./m <sup>2</sup> /min.	
120	Control	—	28.5	67.4	207	290	.324
	41	—	—	106.8	336	474	.318
	60	—	29.6	93.6	310	441	.301
	79	86	—	89.5	231	328	.387
131‡	Control	—	42.5	60.7	177	308	.343
	40	—	36.7	78.6	238	376	.331
	70	—	55.4	64.0	186	437	.344
133	Control	—	42.6	61.5	162	282	.381
	68	—	39.2	103.4	347	572	.295
	95	—	—	15.1	56	94	.268
123	Control	—	41.0	89.2	154	261	.599
	67	—	—	90.0	298	505	.302
	82	57	54.8	29.2	99	218	.295
146	Control	—	35.3	84.5	284	439	.298
	Control	122	36.8	81.0	265	420	.306
	24	140	35.0	85.0	282	434	.302
	48	123	34.9	71.3	217	333	.328
	72	102	38.7	95.5	308	499	.310
	87	68	38.8	41.7	153	250	.273
145	Control	—	34.0	95.8	327	496	.296
	Control	116	37.8	107.6	376	605	.237
	24	115	37.7	97.0	417	670	.232
	48	92	35.7	85.0	319	497	.266
	72	92	38.2	79.8	308	499	.259
147	Control	—	44.8	71.8	204	370	.353
	Control	132	45.0	60.6	166	304	.362
	24	128	47.8	85.2	249	478	.342
	48	124	51.3	75.5	192	395	.391
	72	65	53.0	44.9	144	307	.313
148	Control	136	—	71.5	219	351	.326
	Control	144	37.6	71.3	214	343	.334
	24	108	39.6	111.6	361	598	.310
	48	86	35.7	113.3	416	648	.273

effective renal plasma flow

$$* \text{ Effective renal blood flow} = \frac{1 - \text{hematocrit}}{100}$$

$$\dagger \text{ Filtration fraction} = \frac{\text{filtration rate}}{\text{effective renal plasma flow}}$$

‡ Survivor

study of renal blood flow and filtration rate was therefore undertaken to ascertain if evidence of vasoconstrictor substances is present in dogs intoxicated by HN3.

They attribute these changes to increased renal resistance resulting from afferent arteriolar constriction.

Renal clearances, arterial blood pressure and hematocrit of 8 fasting female dogs determined at 24 hour intervals after intoxication by HN3 are given in Table 2. The renal blood flow increased to a greater or lesser extent at 24 hours or later in all 8 intoxicated dogs, falling to low values only when circulatory collapse occurred and the mean arterial blood pressure had dropped to 70 mm. Hg or below. The filtration fraction did not change significantly during intoxication. With the terminal appearance of circulatory failure, a decrease in renal blood flow occurred with no consistent change in filtration fraction except in one dog (No. 120) in which this figure rose markedly.

The alteration of renal blood flow in these animals is quite the opposite of that observed in tourniquet, hemorrhage or traumatic shock in dogs (26, 27, 28) and in one respect opposed to that observed in traumatic shock (afferent constriction) in man (29). Whereas in tourniquet, hemorrhage or traumatic shock in the dog, renal blood flow is decreased with an increase in filtration fraction in most instances, indicating constriction of the efferent arterioles, in dogs after HN3 intoxication, renal blood flow is increased, indicating renal vasodilatation.<sup>7</sup> The cause of such hyperemia is not known and cannot be attributed to the mannitol used as a diuretic, for the rate of infusion of this substance was only 1.5 cc./min. in both the control and experimental periods. The present data do not permit conclusions regarding the locus of the renal vasomotor change, since the filtration fraction does not change significantly, remaining within the mean normal value of  $\pm 2 \sigma$  (30).

It is evident that progressive reduction in blood volume occurs in many animals intoxicated with HN3, yet despite this fact, arterial blood pressure is maintained within normal limits until the terminal phase, indicating that a compensatory mechanism is operating throughout this period to prevent hypotension. Arbitrarily excluding a possible increase in cardiac output, this compensation may be assumed to be constriction of some large part of the vascular bed, such as is now demonstrated to occur in dogs (26) and man (21) in shock. The present clearance studies give no evidence that the renal circulation participates in this vasoconstriction.

If it is further assumed that the kidneys would necessarily participate in vasoconstriction, were the latter due predominantly to the humoral agents indicated by the experiments of Page *et al* (rather than to neurogenic action), then the present results argue against the presence of such vasoconstrictor humoral agents in dogs intoxicated by HN3. By extension of this argument, no support is obtained for the theory (31, 32) that terminal circulatory collapse is due to exhaustion of either the heart or arteriolar bed in consequence of prolonged bombardment by humoral vasoconstrictor agents. Despite terminal coma and

<sup>7</sup> The fact that the renal clearance of PAH increased rather than decreased after intoxication argues against any decrease in the extraction ratio of PAH, and thus against specific tubular injury by HN3, conforming with the conclusion of Crawford and Smith (22) that HN2 in an LD<sub>50</sub> dose does not specifically injure the excretory mechanism of the renal tubules of rabbits.

hypotension, intoxicated dogs retain cardiovascular responsiveness to adrenalin repeatedly given intravenously.

*Neurologic injury.* No permanent neurologic injury has been demonstrated in dogs intoxicated by the intravenous injection of an  $LD_{50}$  of HN2. Too few dogs have survived the  $LD_{75}$  of HN3 administered by the same route to afford any information on this point.

*White cell counts.* An  $LD_{50}$  of HN2 or an  $LD_{75}$  of HN3 causes a leucopenia which is most marked on the fourth or fifth day after intoxication. The lymphocyte count falls within 24 hours, while the granulocyte count is often increased at this time, decreasing at about 72 hours. In fatally intoxicated dogs, leucopenia is progressive until death. However, the evidence would seem to indicate that this is coincident to rather than the cause of death. Relative to the  $LD_{50}$  of HN2, the dog and rabbit appear equally sensitive to the leucotoxic action. However, leucopenia is apparently not the cause of death in either species (20).

*Gross pathologic changes.* The gross pathologic injury in dogs after intravenous administration of either HN2 or HN3 is essentially the same as in rodents (20). Enteritis is the most notable and consistent change and is generally restricted to the small intestine. The severity of injury ranges from slight congestion with rare petechial hemorrhage to ulcers which nearly penetrate the muscular coat. The serosal surface over these areas is distended and there is a blue-black discoloration. Complete perforation has not been observed. A poor correlation exists between the severity of intoxication, or even the degree of intestinal hemorrhage, and gross changes in the intestinal mucosa. The gastric mucosa sometimes shows congestion grossly, possibly referable to protracted vomiting, atony or reflex dilatation, but hemorrhagic or ulcerative lesions are seldom found in the stomach. The spleen is generally contracted, dry and firm in appearance and hemorrhage is sometimes present. The thymus is variably contracted. The remaining abdominal and thoracic viscera are usually grossly normal.

*Histopathologic changes. Intestinal injury:* In full development, the intestinal injury is revealed as congestion and hemorrhage in the tips of the villi, dilatation of the crypts, which may be plugged with cellular debris, desquamation of the villus epithelium, reduction of the villi to stumps, squamous metaplasia of the epithelium and dilation of the villus lymphatics. The lesion is generally focal, in contrast to that in small animals (20), possibly reflecting variations in the blood supply at the moment of injection of the toxic agent.

*Lymphoid injury.* As shown by the disappearance of lymphocytes from the spleen, thymus and lymph nodes, this is of variable severity at 1.0 mgm./kgm. HN2·HCl but constant and of moderate severity at 2 mgm./kgm. ( $2LD_{50}$ ).

*Myeloid injury.* This is variable but usually moderately severe after 1.0 mgm./kgm. HN2·HCl up to 96 hours. After 2.0 mgm./kgm., the hematopoietic injury is more marked, the femoral marrow being virtually devoid of cells after 100 hours.

*Evaluation of fluid loss. Fluid, electrolyte and colloid therapy:* The results of

subcutaneous fluid and electrolyte on mortality and survival time after HN2 intoxication are given in Table 3. It is apparent that fluid replacement beginning at the time of intoxication and continued during critical illness may reduce the toxicity of an LD<sub>50</sub> of HN2 given intravenously. Glucose and sodium lactate do not seem to be an improvement over saline alone. More impressive than the statistical difference between treated and the untreated series is the dramatic temporary benefit sometimes achieved when saline or saline plus glucose are instituted intravenously in extremely ill and comatose animals, which are frequently brought out of deep coma by this means. That saline alone administered either intravenously or subcutaneously would not be sufficient to effect recovery is, however, indicated *a priori* by persistent intestinal hemorrhage and the probable loss of plasma protein in the diarrhea. Other therapeutic agents such as amino acids, glucose and vitamin B complex have been tested tentatively after intoxication by HN2 without promise of success.

The results of intravenous fluid, electrolyte, and colloid therapy on mortality and survival time after the intravenous injection of 1.0 mgm./kgm. of HN3 are given in Table 3. The number of animals receiving the various forms of therapy is too small to warrant conclusions as to the effect on mortality. However, the data suggest that intravenous infusions may actually be disadvantageous because they aggravate the diarrhea, confirming impressions based on individual animals. There is no pathologic evidence of pulmonary edema from overdosage of saline.

*Occlusion of the circulation to the small intestine.* The circulation to the small intestine of 20 dogs was occluded during and for 15 minutes following the intravenous injection of 1.0 mgm./kgm. of HN3·HCl. All operated dogs were permitted water 12 hours after operation and were offered milk, meat, and dehydrated dog food 24 hours after operation. During the course of intoxication, 6 of 20 dogs received no therapy, while 10 of 20 received subcutaneous saline and 4 of 20 dogs received intravenous Amigen and glucose. Two dogs underwent intestinal clamping for a comparable period, but received no HN3 (operated, normal dogs). Since the post-operative period in these two dogs was uneventful and devoid of symptoms, the clamping procedure *per se* can be eliminated as a contributory factor in the death of the operated intoxicated dogs. The effect of the anesthetic, nembutal, on the course of intoxication cannot be assessed here, for no animals under nembutal were intoxicated without clamping.

Occlusion of the circulation to the small intestine partially protected the ischemic area from the pathologic effects of HN3. The unprotected portions (stomach, duodenum and large intestine) were affected as in non-operated animals. Post mortem examination revealed satisfactory protection of the small intestine and no gross evidence of complication.

*With no therapy.* Six of the 20 operated dogs received no therapy throughout intoxication. In these animals, vascular occlusion was sufficient to prevent the extensive diarrhea and consequent fluid loss from the intestine which characterizes the clinical picture in non-operated animals (Table 1). The average survival time was significantly prolonged, but ultimate mortality was unaffected.

Anorexia and vomiting were occasionally present to a small degree and weight loss was less marked than in unoperated animals at 72 hours after intoxication.

In these 6 dogs, and in the 2 dogs which underwent intestinal clamping for a comparable period but received no HN3 injection (operated, normal dogs), blood volume was determined 24 hours before the operation, and at 24 hour intervals thereafter for three days. Extracellular fluid volume was determined 24 hours before and 72 hours after the operation. Daily red and white cell counts were made. No significant changes occurred in the operated normal dogs. However, in the operated, intoxicated dogs, a parallel reduction of plasma and extracellular fluid volumes and of total circulating plasma protein occurred. The most pronounced change was found within 24 hours after the operation, with a return to approximately the pre-operation level by 48 hours. A further reduction occurred between 72 and 96 hours, even in the absence of diarrhea. At 72 hours, fluid and protein loss was less marked in the operated group than in the unoperated group receiving a comparable dose of HN3 intravenously (Table 1). The clinical appearance in operated animals observed at death was essentially the same as in the non-operated series. A significant leucopenia was present by 72 to 96 hours in the operated, intoxicated dogs, comparable to that seen in unoperated dogs intoxicated by an  $LD_{50}$  of HN2.

*Subcutaneous saline.* Clinical observations alone were made on 10 dogs operated and injected with HN3 and given 800 cc. of isotonic saline subcutaneously per day for at least three days following the operation, together with a 100 mgm. capsule of vitamins A and D and a 100 mgm. tablet of vitamin B complex. In this series, vomiting and diarrhea were more prominent than in operated animals receiving no therapy (Table 1). However, vomiting and diarrhea were not as severe as in unoperated animals. Except for increased fluid loss, the symptoms and pathology of the operated animals were unaltered after subcutaneous saline.

*Parenteral feeding of Amigen and glucose.* Since anorexia and weight loss were present in dogs intoxicated by HN3, the extent to which weight loss could be attributed to inadequate nourishment has remained unsettled, in that intestinal absorption may have been impaired. This suggested a study of animals receiving adequate nutrition by a parenteral route. The daily caloric requirements for this series was estimated at 45 calories/kgm./day. The protein requirement was calculated as 1.0 gram/kgm./day and was supplied by the intravenous infusion of Amigen<sup>8</sup> as a 5 per cent solution in 5 per cent glucose in normal dogs, and as a one per cent solution in glucose in intoxicated dogs. The remaining calories (41 calories/kgm./day) were supplied intravenously by a 25 per cent solution of glucose in the normal dogs, and by an 8 per cent solution in the intoxicated animals. Approximately 4 grams of sodium chloride per day were given in this infusion. All animals received adequate amounts of vitamin B complex<sup>9</sup> intramuscularly. In addition to water *ad libitum* the fluid given par-

<sup>8</sup> Enzymatic casein-pancreas hydrolysate marketed by Mead Johnson Co., Evansville, Indiana.

<sup>9</sup> Obtained through the courtesy of Lederle Laboratories, Pearl River, N. Y.

enterally totalled approximately 100 cc./kgm./day. Body weight was satisfactorily maintained in normal dogs on this regime, and significant weight loss was not present until 24 hours preceding death in intoxicated animals. Vomiting was present and marked in both normal and intoxicated animals receiving Amigen,

TABLE 3

*The effect of parenteral fluid, electrolyte, and protein therapy on mortality and survival time of dogs intoxicated by the intravenous injection of HN2 or HN3*

COM- POUND	DOSE	EXPERIMENTAL PROCEDURE	THERAPY	NO. OF DOGS	PER CENT DEATHS	SUR- VIVAL TIME
	mgm./kgm.					(hours)
HN2	2.0	Intoxicated	saline and glucose, s.c.* or sodium lac- tate s.c.	8	87.5	104
	1.0		none	10	50.0	130
			saline, s.c.	14	28.5	127
			saline and glucose, or saline and sodium lactate, s.c.	5	80.0	96
			sodium lactate, s.c.	10	50.0	166
HN3	1.0	Intoxicated	none	16	75.0	76
			5% bovine albumin and 5% glucoss in saline i.v.†	4	100.0	89
			acacia in glucose, i.v.	4	100.0	86
			acacia in saline, i.v.	6	83.0	93
			human plasma‡ i.v.	3	100.0	82
			human plasma and saline, i.v.	3	100.0	124
			dog plasma, i.v.	5	60.0	102
			8% gelatin,§ i.v.	2	50.0	84
			8% gelatin and 5% glucose in saline, i.v.	3	100.0	104
				Operated,‡ Intoxicated	none	6
	800 cc. saline s.c./day	10			90.0	106
	5% Amigen in 5% glucose, i.v.	4			100.0	94

\* s.c. = subcutaneous injection.

† i.v. = intravenous injection.

‡ operation = occlusion of circulation to small intestine during and for 15 minutes after the intravenous injection of HN3·HCl.

§ "Lyovac" = rapidly lyophilized normal human plasma was obtained from Sharp and Dohme, Philadelphia, Pa.

|| Administered as an 8 per cent solution from Edible Gelatin Manufacturers Research Society of America, Inc. Lot No. 87.

though diarrhea was absent. Survival time in the latter was not prolonged and mortality was unaffected (Table 3). A marked fall in plasma volume and total circulating protein occurred in 2 of 4 animals examined (Table 1). These 2 dogs died within an hour of the determination. The 2 animals in which no significant change in these values occurred survived 24 to 48 hours after the determination.

## SUMMARY AND CONCLUSIONS

Dogs were intoxicated by the single intravenous injection of 1.0 mgm./kgm. of the hydrochloride of methyl-*bis*( $\beta$ -chloroethyl)amine (HN2 HCl) or *tris*( $\beta$ -chloroethyl)amine (HN3·HCl). Mortality, survival time, changes in blood and plasma constituents and properties, changes in body water compartments and renal function were studied with the intent of ascertaining the cause of death.

Vomiting accompanied by anorexia begins within a few hours after intoxication, increasing in severity and generally continuing through the second and third day. Diarrhea, usually blood-stained or frankly hemorrhagic, is generally present on the second to fourth days.

Biochemical and water balance data reveal reduction in volume of both extracellular fluid and circulating plasma, reduction in plasma chloride concentration, increase in carbon dioxide capacity and blood pH, reduction of total circulating plasma protein, increase in concentration of plasma protein, indicating a greater loss of plasma water than protein, a variable reduction in total circulating red cell volume which may be accounted for by intestinal hemorrhage or possible *in vivo* sequestration (although this has not been indicated in this study), a variable increase in hematocrit, and a variable increase in hemoglobin or oxygen capacity. These changes are at least in part attributable to profuse vomiting and diarrhea.

Reduction in body weight results from excessive fluid and protein loss and is more extensive than that due to starvation alone.

Terminal weakness and coma, preceding death, occur in the majority of dogs on the third to fifth day after intoxication and are associated with low mean femoral arterial blood pressure, marked oxygen unsaturation of jugular blood (with presumably normal arterial oxygen saturation), reduction in body temperature, coldness of extremities, relaxation of the anal sphincter, and respiratory failure.

Excluding a few animals with severe pulmonary injury or infection as a complication, it is inferred that death is caused by anoxia of the respiratory centers as a consequence of peripheral circulatory failure precipitated chiefly by a reduction in blood volume attributable to loss of proteins, electrolytes, and water through vomiting and diarrhea, supplemented by loss of red cells through as yet unidentified channels. Undescribed pathologic changes may contribute to a fatal effect. No support is obtained for the theory that terminal circulatory collapse is due to exhaustion of either the heart or arteriolar bed, in consequence of prolonged bombardment by humoral vasoconstrictor agents. Despite terminal coma and hypotension, intoxicated dogs retain cardiovascular responsiveness to adrenalin given intravenously.

No evidence was found for renal vasoconstriction during intoxication. Renal blood flow, as judged by the clearance of p-aminohippuric acid at low plasma levels, actually increased in dogs at 24 hours or later. However, terminally when circulatory failure occurred, renal blood flow decreased to low values, with no consistent change in filtration fraction.



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Two general methods have been used in an attempt to evaluate the significance of fluid loss in intoxicated animals. 1) Replacement of fluid, electrolyte, and protein by various methods, although apparently prolonging life in some instances, has proved unsuccessful in preventing death. Under such therapy, diarrhea was aggravated. 2) Protection of the small intestine by occlusion of the circulation during and for 15 minutes after the intravenous injection of the toxic agent prevented the extensive diarrhea and consequent fluid loss from the intestine, and prolonged the average survival time slightly, but without affecting the ultimate mortality. Parenteral feeding of these "protected" animals with Amigen and glucose did not alter survival time or mortality, although diarrhea was absent, and loss of body weight was reduced.

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# THE ACTION OF THE BASIC AMINO ACIDS ON THE HEART AND INTESTINE

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Despite the extensive investigations concerning the nutritional and metabolic aspects of the amino acids, few data are available on their pharmacodynamic action. Kondo (1, 2) reported that arginine inhibited contractions of the isolated frog or toad heart but markedly potentiated the action of epinephrine. The inhibitory action of arginine was confirmed by Sigg (3), who studied the effects of various amino acids and amines on the frog and the frog heart. Arginine or histidine alone was without effect on the vascular tone of the perfused rabbit ear (4), but increased the vasoconstrictor action of epinephrine (5, 6, 7). Sensitivity of the vascular system to histamine was reduced by arginine according to Ackermann (8), however, Rocha e Silva (9) was unable to confirm this observation. The latter investigator noted that histidine per se was hypotensive.

Arginine or histidine undoubtedly inhibits the action of histamine on the isolated intestine and uterus, but the influence of these amino acids on acetyl choline action has been found to be variable (10-14). However, Mackay (15), Credner and Schumrick (16) and Rocha e Silva (9) reported arginine and histidine as effective inhibitors of acetyl choline and histamine in the isolated intestine.

The present communication deals with the action of the basic amino acids 1 (+) histidine, 1 (+) arginine and 1 (+) lysine on the heart and intestine. The amino acid solutions were prepared from the monohydrochloride salts and all concentrations are expressed on this basis.

**EFFECT ON THE ISOLATED FROG HEART.** Isolated frog hearts were prepared according to the technique of Straub. All amino acid stock solutions used in this series of experiments were adjusted to pH 7.4. The addition of arginine or lysine to the Ringer's solution in the standpipe immediately depressed the heart action. On the other hand histidine caused a slight increase in the tone and amplitude. Concentrations as low as 0.7% arginine or 1.0% lysine effected a transient decrease in the amplitude with no significant change in the heart rate or tone (figure 1). Higher concentrations accentuated the depressant action of arginine and lysine and at certain threshold values resulted in complete diastolic standstill. These minimal paralyzing concentrations (M.P.C.) were as follows: arginine 1.5% (0.071 M.) and lysine 4.5% (0.25 M.). In contrast to these inhibitory effects histidine caused a slight improvement of the tone and amplitude even at concentrations as high as 4% (0.21 M., figure 2). The depressant action

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of arginine and lysine was reversible for the normal beat returned when the amino acid solution was replaced by fresh Ringer's solution.

The stimulating action of ouabain (50  $\gamma$  per ml.) or epinephrine (1  $\gamma$  per ml.) gave way to an immediate paralysis on addition of the M.P.C. of arginine or

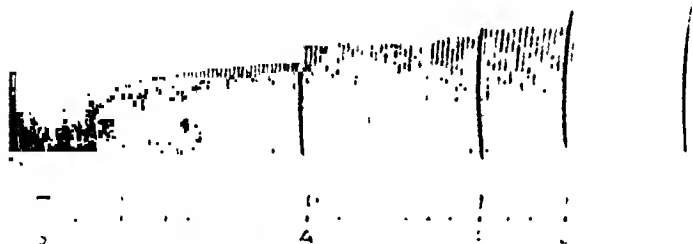


FIG. 1. Action of lysine HCl 1.5% solution (pH 7.1) on the isolated frog heart, given at 3 Time interval 10 seconds. At: A, B, C the kymograph was stopped for 3 minutes; D for 6 minutes.

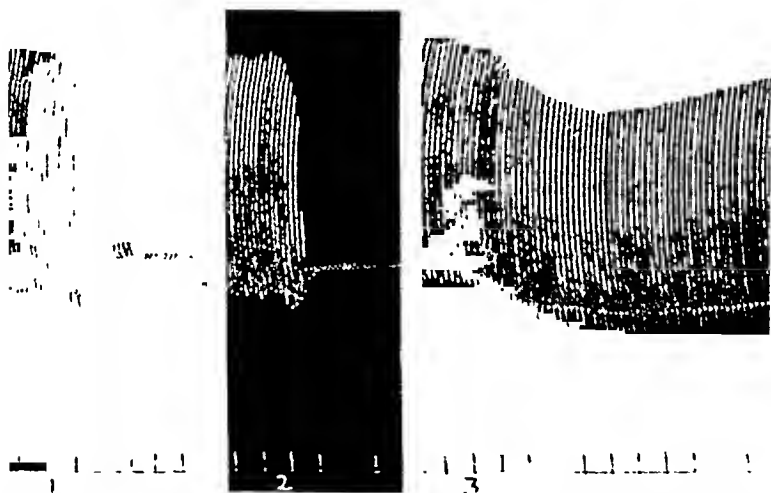


FIG. 2. Action of basic amino acids at pH 7.4 on the isolated frog heart. Time interval 10 seconds. At: (1) 2% arginine (pH 7.4); (2) 5% lysine (pH 7.4); (3) 0.2% histidine (pH 7.4).

lysine. On prolonged contact with arginine or lysine at concentrations slightly below M.P.C., the heart "escaped" from the inhibition with subsequent decreased rate and increased amplitude (figure 1). When the amino acid solution was removed from an "escaped" heart and tested on a fresh heart preparation, an immediate inhibition resulted indicating that no significant change in the depressant activity of the solution had occurred. Experiments with sodium chloride and with glucose solutions equi osmotic with those of the basic amino

acids indicated that the observed effects were not a result of "salt action". Treatment of the heart with atropine (50  $\gamma$  per ml.) did not inhibit the depressant action of the amino acids although sensitivity to mechohyl (acetyl- $\beta$ -methylcholine chloride) was abolished.

**EFFECTS ON BLOOD PRESSURE AND HEART IN SITU.** For the experiments on blood pressure, heart rate and heart action, cats were anesthetized with sodium pentobarbital (35 to 40 mg. per kg) intraperitoneally. Carotid blood pressure was recorded in the usual way using mercury manometer. Changes in heart rate were recorded graphically as described by Kniazuk and Unna (17). All solutions were administered by injection into the femoral vein

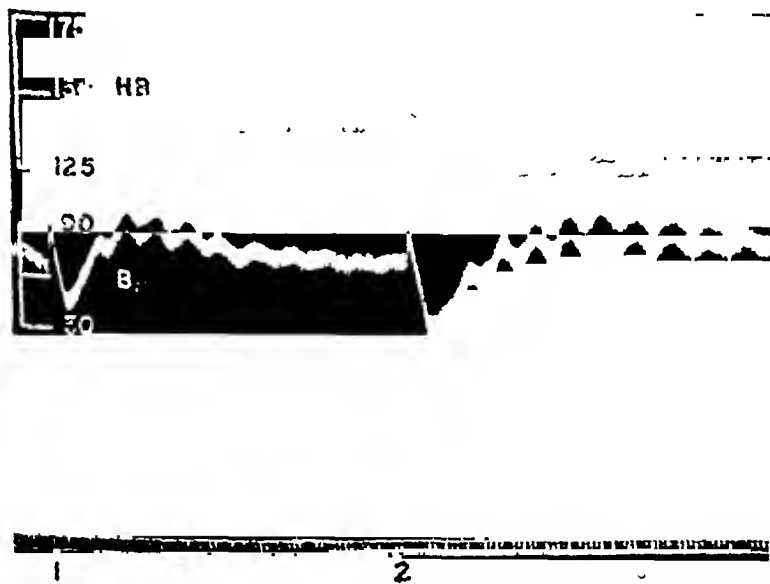


FIG. 3. Effect on blood pressure (Bp) and heart rate (HB, beats per minute) of anesthetized cat due to lysine or arginine administered at pH 7.4. Time interval 10 seconds. At: (1) lysine and (2) arginine, 475 mg/kg I V

In certain experiments the heart action was recorded by a Cushny myocardiograph attached to the right ventricle, pulmonary ventilation being maintained with a respiration pump. In order to record the heart rate in these experiments, the pick-up electrode of the recorder was attached directly to the myocardiograph.

In the anesthetized cat, arginine or lysine administered rapidly as a 15% solution buffered to pH 7.4 had a hypotensive action accompanied by a simultaneous decrease in the heart rate and amplitude (figure 3). Histidine, at the same pH, resulted in a slight rise in blood pressure with no apparent action on the heart (figure 4). However, as the monohydrochloride, histidine (pH 4.0) as well as arginine (pH 5.6) and lysine (pH 5.5) exhibited these hypotensive and cardio-depressor effects (figure 5). Atropine sulfate (0.2 mg per kg), intra-

venously, did not alter the action of the basic amino acids on the heart or blood pressure.

Arginine and lysine (monohydrochloride or at pH 7.4) in doses of 400 to 500 mg. per kg. caused a sharp decline in blood pressure of about 50 mm. Hg with a coincident reduction of the cardiac rate and amplitude (figures 3, 5). Arginine had a more marked effect on the heart muscle than lysine and caused observable dilatation with a decrease of 40% in the amplitude and 15% in the rate. The return of the heart action and the blood pressure to normal was delayed for as long as eight minutes during which time Traub-Herring waves frequently appeared. Lysine caused a mild dilatation of the heart and only a slight reduction

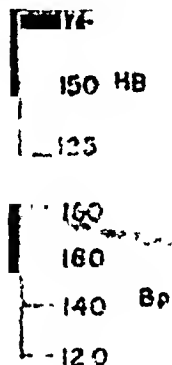


FIG. 4. Effect on blood pressure (Bp), heart rate (HB, beats per minute) of anesthetized cat due to histidine administered at pH 7.4 or 4.0. Time interval 10 seconds. At: (1) histidine (pH 7.4), 210 mg./kg., I.V.; (2) histidine (pH 4.0), 180 mg./kg., I.V.

in the cardiac rate and amplitude. Recovery after lysine injection required only one to three minutes.

With histidine hydrochloride, 50 to 175 mg. per kg. resulted in a 50 mm. fall in blood pressure followed by a rapid return to normal. At the higher doses an extreme bradycardia and reduction in amplitude occurred (figure 5). In fact on direct observation it was noted that the heart stopped with marked dilatation for about five seconds after administration of this amino acid. (Since the heart rate recorder counts for ten second cycles, this severe condition could not be recorded graphically.) Recovery of the heart was quite rapid, paralleling that of the blood pressure. As mentioned previously, these effects were not observed when histidine was administered at pH 7.4 (base). A comparison of the action of histidine as the base and monohydrochloride is given in figure 4.

Blood pH<sup>1</sup> and respiration were recorded in conjunction with several of these experiments on the blood pressure and heart rate. Injection of the monohydrochloride of arginine, lysine or histidine caused a slight reduction in blood pH (0.20 to 0.25 units) and the usual fall in blood pressure and heart rate. In addition, histidine HCl markedly increased the respiratory volume but lysine HCl or arginine HCl caused little change. When the basic amino acids were administered in a solution buffered to pH 7.4 no change in respiratory volume or blood pH was noted and histidine showed no hypotensive or bradyeardial action.

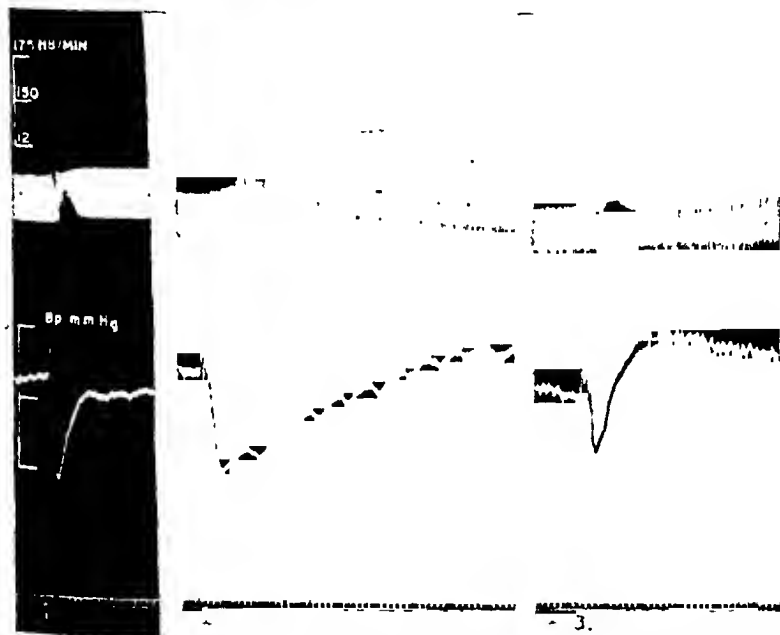


FIG. 5. Effects of basic amino acids on blood pressure (Bp), heart rate (HB, beats per minute) and heart action of anesthetized cat. Time interval 10 seconds. At: (1) histidine HCl, 140 mg./kg. (pH 4.0) I.V.; (2) and (3) arginine HCl (pH 5.6) and lysine HCl (pH 5.5), respectively, 375 mg./kg., I.V.

Hydrochloric acid at the same molar concentration as that of the 15 per cent monohydrochloride solution (0.79 M, pH < 1) caused no significant change in the blood pressure, heart rate or respiration but decreased the blood pH to the same extent as did the amino acid salts.

EFFECT OF SLOW INFUSION ON BLOOD PRESSURE AND HEART ACTION IN SITU. Histidine HCl was used in all intravenous infusion studies. A total of 400 mg. per kg. was administered as a 4.05% solution by means of an infusion pump at rates varying from 25 to 145 mg. per kg. per min., requiring from 2.6 to 16 min-

<sup>1</sup> The blood pH was recorded by a special glass electrode placed in the inferior vena cava slightly above the level of the kidneys.



utes for administration. Individual animals showed marked variations in the blood pressure response, but changes in the heart rate and action were similar at like infusion speeds. Administered at the rate of 25 mg. per kg. per min., histidine HCl had no apparent effect on the blood pressure or cardiac rate. Increasing the rate of administration above this point resulted in depression of the cardiac rate and action and frequently the blood pressure (figure 6). Decreased amplitude accompanied by dilatation of the heart was the first sign of cardiac depression and at the faster infusion rates appeared within 30 seconds. Bradycardia became evident shortly thereafter, but this interval may be an artifact when one considers the time cycle of the heart rate counter. When a fall in blood pressure occurred, it was usually observed shortly after the cardiac effects were initiated. Depression persisted for the duration of the infusion and could not be antagonized by 10  $\gamma$  of epinephrine administered intravenously. After

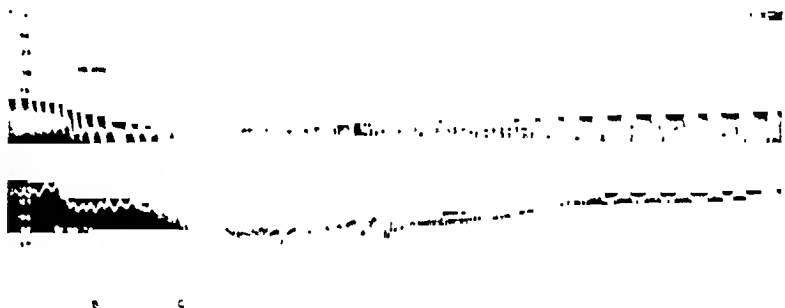


FIG. 6. Effect of infusion of histidine HCl on blood pressure (Bp), heart action, and heart rate (HB, beats per minute) of anesthetized cat. Time interval 10 seconds. At: (A)-(C) infusion of histidine HCl (pH 4.0), 143 mg./kg. per minute (total 400 mg./kg.). (B and C) 10  $\gamma$  epinephrine I.V. (infusion started 45 seconds before A).

the administration of histidine HCl was discontinued, seven or more minutes were required for the maximum recovery of the cardiac rate, amplitude and tone. However, the heart did not recover completely, indicating a more than transitory inhibition or weakening of the musculature. Atropinization did not alter the effects of the histidine HCl infusion.

**EFFECTS ON THE ISOLATED INTESTINE.** In the studies on the isolated intestine of the rabbit the technique of Magnus was used. The duodenal segment (3 cm.) was suspended in continuously oxygenated Locke-Ringer's solution (pH 7.8) maintained at a temperature of 39°C. Mecholyl (0.02  $\gamma$  to 0.1  $\gamma$  per ml.) was used as a parasympathetic nervous system stimulant while barium chloride (25  $\gamma$  to 50  $\gamma$  per ml.) served as a muscle stimulant. These agents produced a like spasm at the aforementioned concentrations. Mecholyl could easily be removed by rinsing, permitting several tests to be run on the same strip but barium chloride was difficult to wash out and a new strip was required for each test. All amino acid stock solutions were adjusted to pH 7.4 and warmed before adding to the test bath.

Arginine, histidine or lysine at a concentration of 0.75 to 1.5% lowered the tone of normal intestinal strips and inhibited or counteracted the contractions produced by mecholyt or barium (figure 7). No effect on the rhythmic contractions was noted. It was indicated that these amino acids also reduce the sensitivity of the intestine to histamine. Arginine and lysine appeared somewhat more effective than histidine in lowering the tone of the duodenal segment. Apparently the inhibitory effects are reversible because on washing with fresh Locke-Ringer's solution the segment returned to normal length and demonstrated a normal sensitivity to barium or mecholyt. The basic amino acids were slightly more effective in inhibiting the mecholyt effect than the barium effect. Addition of an amino acid to the bath enhanced the relaxing effect of epinephrine (0.1  $\gamma$  to 1  $\gamma$  per ml.). Atropine at a concentration that counteracted the contractions due to mecholyt (0.01  $\gamma$  to 0.1  $\gamma$  per ml.) had no effect on those due to

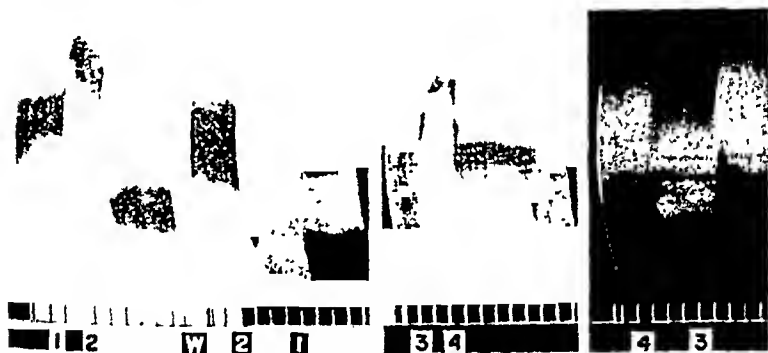


FIG. 7. Action of arginine and lysine on isolated rabbit duodenum. Time interval 1 minute. At: (1) mecholyt 0.01  $\gamma$ /ml., (2) 1.13% arginine (pH 7.4); (3) barium chloride 50  $\gamma$ /ml., (4) 0.75% lysine (pH 7.4); W-wash. New strips were used for each experiment.

barium, but an immediate relaxation was effected by addition of any of the basic amino acids.

**EFFECT ON THE INTESTINE IN SITU.** Fasted rabbits anesthetized with 30 mg. per kg. of nembutal were used for these experiments. A combination of the balloon and mechanical lever methods permitted separate recording of circular and longitudinal contractions. For recording circular contractions a balloon about 4 cm. in length was introduced into the duodenum through an incision in the pyloric region and passed caudal to a position about 15 cm. down the length of the duodenum. Longitudinal contractions were recorded with a modified form of Jackson's "internal organ apparatus" (18) attached to a 2 cm. segment of the duodenum slightly below the end of the balloon. A metal trough, from the instrument covered this segment and eliminated the possibility of artifacts due to the movements of the abdominal viscera. Tension was maintained by a light weight on the recording heart lever. All test solutions were administered at 15% concentration directly into the jugular vein. The effects of barium chloride and mecholyt were used as controls for increased tonus and motility of the gut.

Arginine and lysine injected as the monohydrochloride or at pH 7.4 relaxed

the segment, decreased the tonic *longitudinal* contractions and completely inhibited contractions of the *circular* muscles. Histidine at this pH had no apparent effect on the intestinal musculature *in situ* in contrast to the inhibition observed in the isolated segment. On the other hand, the monohydrochloride of histidine showed an action similar to that observed for arginine or lysine but caused a short spasm of the *circular* muscles.

Following the administration of 250-300 mg. per kg. of arginine or lysine there was an immediate elongation of the duodenal segment and a reduction in the tonic *longitudinal* contractions. Normal tone and contraction returned in from 8 to 10 minutes. The *circular* muscles were paralyzed and this effect persisted for the duration of the experiment. Even after barium stimulation (5 to 10 mg. per

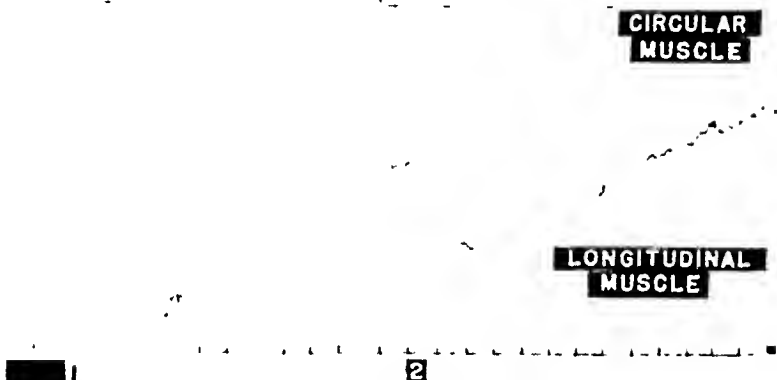


FIG. 8. Action of histidine HCl and arginine HCl on intestine *in situ* of anesthetized rabbit. Time interval 1 minute. At: (1) 100 mg./kg. histidine HCl (pH 4.0) I.V. (2) 250 mg./kg. arginine HCl (pH 5.6) I.V.

kg.), arginine and lysine rapidly paralyzed the *circular* muscles and reduced slightly the tonic contractions and tone level of the *longitudinal* muscles.

Histidine HCl, in doses of 50 to 100 mg. per kg. relaxed the *longitudinal* muscles and reduced their tonic activity as did arginine and lysine (figure 8). At the same time there was a spasm of the *circular* muscle lasting for about 15 seconds. No apparent effect on the rate or height of peristaltic contractions was noted. After barium, histidine HCl relaxed the *longitudinal* muscles slightly but did not affect the heightened activity of the *circular* muscles.

When the dose levels of the basic amino acids used in this series of experiments inhibited the intestinal activity, cardiac action was also depressed as evidenced by simultaneous recording of the heart rate and the intestinal activity.

DISCUSSION. Arginine and lysine were found to depress the *isolated* frog heart while histidine exerted a stimulating action. These findings are in accordance

with those reported by Sigg (3) and Kondo (1, 2). However, the latter author found that arginine acts synergistically with epinephrine in strengthening the beat although when administered alone it paralyzes the heart. In our experiments arginine and lysine antagonized the action of epinephrine on the isolated heart. In both the *isolated* and *in situ* experiments it was indicated that these amino acids probably act directly on the heart and blood vessels since their action was not abolished by atropinization.

Studies on the intestine of the rabbit *in situ* revealed a fundamental difference between the action of histidine HCl and the other two basic amino acids. Although they all relaxed the *longitudinal* muscles of the duodenum, histidine HCl caused a spasm of the *circular* muscles and did not affect the rhythmic contractions while arginine and lysine inhibited all activity of the *circular* muscles. This "splitting action" noted for histidine HCl has recently been demonstrated with several compounds of a pyrrolidine series by Loomis and Schaffer (19) and with morphine by Trendelenberg in 1917 (20).

The difference in the action of arginine and lysine as compared with histidine on the isolated heart might be interpreted as a "specific" effect of these compounds. However, another factor to consider is the amphoteric nature of the amino acid molecule. Since each of the basic amino acids has a different isoelectric point (arginine pH 10.8, lysine pH 9.7 and histidine pH 7.6) the state of ionization of the basic and acidic groups of each will differ at a given pH. Arginine or lysine at pH 7.4 is on the acid side of its isoelectric point and acts as a cation. Histidine at this pH approximates its isoelectric point and therefore acts as a "zwitterion".

The experiments on the *isolated* intestine do not follow those on the *isolated* heart because the *isolated* intestine is relaxed by histidine in solution at pH 7.4. Roche e Silva (9) and Ackermann and Wasmuth (21) suggested that the imine ( $=NH$ ) group of histidine and arginine must be free to inhibit the action of histamine on the isolated intestine. It is assumed that the imine group of these amino acids competes with the like group of histamine for certain chemical receptors of smooth muscle. This, however, does not explain the inhibition of mecholyt action by the amino acids nor their synergism with epinephrine in the isolated intestine.

*In situ*, histidine at pH 7.4 had no significant effect on the blood pressure, heart or intestine but as the monohydrochloride salt (pH 4.0), a marked depression was observed. In view of the slight decrease in the blood pH (0.25 units) it might be assumed that the actions attributed to histidine HCl are a result of acidosis. The following observations do not appear to support this premise: (1) only histidine HCl caused an increase in respiratory volume; arginine and lysine had no apparent effect at the same pH as the histidine HCl solution (pH 4.0), (2) hydrochloric acid at the same molar concentration as the histidine hydrochloride solution (0.79M., pH <1) was devoid of any blood pressure, cardiac or respiratory effect, but did decrease the blood pH to the same extent as the amino acid solution, (3) arginine and lysine in solutions at pH 7.4 had the same hypotensive, bradycardial and intestinal relaxing action as noted with the hydro-

chloride salts, but caused no change in blood pH. An excess of ionized basic groups does not seem an adequate explanation for the change in the action of histidine in the *in situ* experiments because even when histidine salt at pH 4.0 is injected, the base is rapidly formed on contact with the buffer systems of the blood and reaches the tissue as the base.

The fact that histidine solutions do not cause a fall in blood pressure at pH 7.4 precludes the possibility of histamine contaminations for the latter is depressant at that hydrogen ion concentration.

#### SUMMARY AND CONCLUSIONS

1. Arginine and lysine at pH 7.4 paralyze the isolated frog heart and antagonize the stimulating action of ouabain and epinephrine. Histidine has a slight stimulating action.

2. Intravenous injection of arginine or lysine at pH 7.4 or as the monohydrochloride (pH 5.6 and 5.5 respectively) depresses the heart rate and action as well as the blood pressure in the anesthetized cat. At pH 7.4 histidine (base) has no apparent action, but as the monohydrochloride salt (pH 4.0) exhibits the same depressant action as arginine and lysine.

3. Slow intravenous infusion of histidine HCl into the cat causes a gradual decline of the heart rate and action and frequently the blood pressure.

4. These amino acids appear to act directly on the muscles of the heart and blood vessels.

5. In the *isolated* rabbit duodenum the basic amino acids at pH 7.4 reduce the tone of the *longitudinal* muscles but do not affect the rhythmic contractions.

6. *In situ*, the *longitudinal* duodenal muscles of the rabbit are relaxed and peristalsis inhibited by arginine and lysine at pH 7.4 or as the monohydrochloride. Histidine base (pH 7.4) has no such effect, however, the monohydrochloride injection results in a decreased *longitudinal* tone and a short spasm of the *circular* muscles.

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# THE INFLUENCE OF RATE OF ADMINISTRATION UPON THE LETHAL DOSE OF CARDIAC GLYCOSIDES<sup>1</sup>

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The influence of rate of administration on the lethal dose of some pure and galenic preparations of cardiac glycosides has been studied with controversial results. Some investigators could detect no alteration of the lethal dose of cardiac glycosides when rate of administration or experimental time was varied under otherwise similar conditions (1-5). Other studies (4, 6-15) have shown a marked influence of rate of administration upon the lethal dose. Thus Kingsepp and Lendle (4) demonstrated that an increase in the experimental time from one hour to two hours resulted in a 50 per cent decrease of the lethal dose of digitoxin.

The only systematic studies on the effect of rate of administration on the lethal dose of cardiac glycosides were carried out in cats by Hildebrandt (13), who used crystalline digitoxin "Merek" and g-strophanthin, and by Matuda et al (11), and Bliss and Allmark (14), who worked with tincture of digitalis. The object of the present study was to confirm and extend Hildebrandt's studies.

**MATERIALS AND METHODS.** Crystalline digitoxin<sup>3</sup>, digoxin<sup>4</sup>, and oleandrin<sup>5</sup> were used in the present study. The following molecular weights were used in the conversion of the data to molar equivalents: digitoxin 764.5; digoxin 780.9; and oleandrin 576.7. In converting the milligram values of g-strophanthin obtained by Hildebrandt (13) this glycoside was assumed to have 11 per cent water of crystallization and a molecular weight of 648.7.

Dilutions were made at the time of the experiment in 0.9 per cent sodium chloride solution from a stock solution containing 1 mgm of the glycoside per cc of 95 per cent ethyl alcohol. The millimolar concentration ranges used were: for digoxin, 0.174 to 0.0154; for oleandrin, 0.0653 to 0.0185; and for digitoxin 0.0563 to 0.0115. No precipitation occurred at these concentrations; erroneous results will be obtained if this point is not carefully controlled.

Male and non-pregnant female cats weighing between 1.75 and 3.25 kilograms were used. The influence of anesthesia upon the lethal dose of cardiac glycosides has been recently reviewed by Holek, Smith, and Shuler (16). They have shown the lethal dose with urethane to be 20 per cent greater than with ether, while with dial and pentobarbital the lethal dose was 51 and 47 per cent greater respectively than with ether. Kaplan and Visseher (17) have found the lethal dose of Lanatoside C in the unanesthetized animal and the animal under pentobarbital anesthesia to be statistically the same; however, they made the important

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<sup>3</sup> Kindly supplied by Drs. R. P. Linstead and W. S. Johnson, Department of Chemistry, Harvard University.

<sup>4</sup> Kindly supplied by Wellcome Research Laboratories, Tuckahoe, New York.

<sup>5</sup> Kindly supplied by the Schering Corporation, Bloomfield, N. J. and marketed as *Olnerin*. Known also as *Folinerin* in some foreign countries.

observation that the lethal dose under these conditions was 41 to 50 per cent higher than the lethal dose obtained under ether anesthesia. The data of Hildebrandt with digitoxin and g-strophanthin were obtained under anesthesia with butyl,  $\beta$ -bromallylbarbituric acid (pernocton). In the present series of experiments the anesthetic used was diallylbarbituric acid (Dial-Ciba)<sup>6</sup> given in a dose of 0.09 to 0.12 grams per kilogram intraperitoneally.

The animals were placed upon a heated table and the rectal temperature was recorded. Artificial respiration was administered by means of a Starling respiration pump. The femoral vein was cannulated to permit injection of drugs, and the blood pressure was recorded from the carotid artery by means of an Anderson glass membrane manometer (18) or a mercury manometer. Heparin,<sup>7</sup> 3-4 mgm per kgm, was used as an anticoagulant. Heparin has been shown not to alter the lethal dose of tincture of digitalis in the cat by Ramsey, Pinschmidt and Haag (19).

The electrocardiogram was recorded by means of a Grass ink-writing oscillograph leading off from the left hindleg and the right foreleg. Records were taken every 3-5 minutes throughout the experiment. In calculating the irregularity dose, the appearance of ventricular premature beats, progressing to idioventricular rhythm, was taken as the onset of irregularity. Electrocardiographic evidence of ventricular fibrillation, that is, rapid ir-

TABLE 1  
*Digoxin*

NUMBER OF EXPERIMENTS	RATE OF ADMINISTRATION	LETHAL DOSE	EXPERIMENTAL TIME	IRREGULARITY DOSE (% OF LETHAL DOSE)
	<i>Micromols/Kgm/Hour</i>	<i>Micromols/Kgm</i>	<i>Minutes</i>	
4	2.68	0.800 $\pm$ 0.101	18 $\pm$ 2.3	76 $\pm$ 3.1
4	1.98	0.767 $\pm$ 0.033	23.5 $\pm$ 1.3	68 $\pm$ 1.7
8	1.30	0.685 $\pm$ 0.025	31.5 $\pm$ 1.4	74 $\pm$ 1.0
4	0.645	0.498 $\pm$ 0.061	46.5 $\pm$ 5.6	80 $\pm$ 2.5
7	0.424	0.378 $\pm$ 0.033	53 $\pm$ 4.7	84 $\pm$ 2.0
4	0.226	0.300 $\pm$ 0.029	80 $\pm$ 8.0	83 $\pm$ 2.0
4	0.128	0.275 $\pm$ 0.034	129 $\pm$ 17.3	81 $\pm$ 2.2
4	0.068	0.239 $\pm$ 0.016	211 $\pm$ 14.7	88 $\pm$ 2.4
3	0.048	0.240 $\pm$ 0.014	354 $\pm$ 21.8	82 $\pm$ 7.7

regular sine wave motion of the recording device, associated with a fall in blood pressure to zero, was taken as the end of the experiment.

After a control period the infusion of the cardiac glycoside was begun through the cannula in the femoral vein. These infusions were made through a calibrated 50 cc syringe, the plunger of which was driven by a constant infusion pump.<sup>8</sup> The volume of the infusion per hour and the concentration of the solution infused were varied to achieve the desired rate of administration expressed in micromols of the glycoside per kilogram per hour.

RESULTS. The results of the infusion of cardiac glycosides into 105 cats have been analyzed. Of these 44 experiments were with oleandrin, 42 with digoxin, and 19 with digitoxin. The results are presented in tables 1-3. In the last column is given the irregularity dose for each rate of administration expressed in per cent of the lethal dose.

<sup>6</sup> Kindly supplied by Ciba Pharmaceutical Products, Inc., Summit, New Jersey.

<sup>7</sup> Kindly supplied by Eli Lilly and Company, Indianapolis, Indiana.

<sup>8</sup> Constructed by K. Kniazuk, Merck Institute for Therapeutic Research, Rahway, New Jersey.



In figure 1 the rate of administration has been plotted against the lethal dose. For comparison the data of Hildebrandt (13) obtained with g-strophanthin and digitoxin have been included. It can be seen from figure 1 that the lethal dose of all the glycosides varies considerably with the rate of administration. At high rates of administration the lethal dose is high, at low rates it progressively decreases until at very low rates of administration it tends to become constant. The constant value for the lethal dose of every glycoside which is obtained at the lower rates of administration will be referred to as the

TABLE 2  
*Oleandrin*

NUMBER OF EXPERIMENTS	RATE OF ADMINISTRATION	LETHAL DOSE	EXPERIMENTAL TIME	IRREGULARITY DOSE (% OF LETHAL DOSE)
	<i>Micromols/Kgm/Hour</i>	<i>Micromols/Kgm</i>	<i>Minutes</i>	
4	3.513	$1.302 \pm 0.057$	$22 \pm 1.0$	$70 \pm 8.7$
4	1.962	$0.875 \pm 0.118$	$27 \pm 3.7$	$71 \pm 4.6$
4	0.798	$0.589 \pm 0.083$	$44 \pm 6.2$	$69 \pm 6.3$
4	0.546	$0.511 \pm 0.021$	$56 \pm 5.2$	$83 \pm 2.8$
4	0.312	$0.434 \pm 0.021$	$83.5 \pm 4.0$	$77 \pm 2.8$
4	0.169	$0.320 \pm 0.010$	$114 \pm 3.7$	$82 \pm 1.0$
4	0.092	$0.300 \pm 0.013$	$195 \pm 8.5$	$87 \pm 3.3$
4	0.059	$0.273 \pm 0.020$	$276 \pm 20.5$	$86 \pm 3.7$
8	0.044	$0.213 \pm 0.014$	$295 \pm 21.4$	$81 \pm 2.2$
4	0.034	$0.221 \pm 0.013$	$392 \pm 21.5$	$86 \pm 2.8$

TABLE 3  
*Digitoxin*

NUMBER OF EXPERIMENTS	RATE OF ADMINISTRATION	LETHAL DOSE	EXPERIMENTAL TIME	IRREGULARITY DOSE (% OF LETHAL DOSE)
	<i>Micromols/Kgm/Hour</i>	<i>Micromols/Kgm</i>	<i>Minutes</i>	
4	0.607	$0.897 \pm 0.070$	$89 \pm 7.1$	$74 \pm 3.3$
3	0.362	$0.654 \pm 0.026$	$108 \pm 4.4$	$81 \pm 5.5$
4	0.166	$0.544 \pm 0.018$	$197 \pm 5.5$	$85 \pm 3.3$
4	0.081	$0.336 \pm 0.017$	$397 \pm 7.7$	$75 \pm 3.7$
4	0.0334	$0.313 \pm 0.010$	$564 \pm 19.1$	$80 \pm 7.1$

*minimal lethal dose* (M.L.D.). Furthermore, it is clear from figure 1 that each cardiac glycoside has a characteristic rate of administration at which the lethal dose tends to become constant. The characteristic highest rate of administration at which the M.L.D. can still be obtained has been called the *optimal rate of administration* (15).

A reduction in the rate of administration always results in a prolongation of the experimental time. It is obvious therefore that each cardiac glycoside studied has a characteristic minimal experimental time at which the M.L.D. is determined; we call this the *optimal experimental time*. In figure 2 the relationship of experimental time to lethal dose is illustrated.

In table 4 the values for the optimal rate of administration, optimal experimental time, and the M.L.D. have been tabulated for g-strophanthin, digoxin, oleandrin, and digitoxin. On a molar basis g-strophanthin is about twice as potent as digitoxin and about one and one half times as potent as oleandrin and

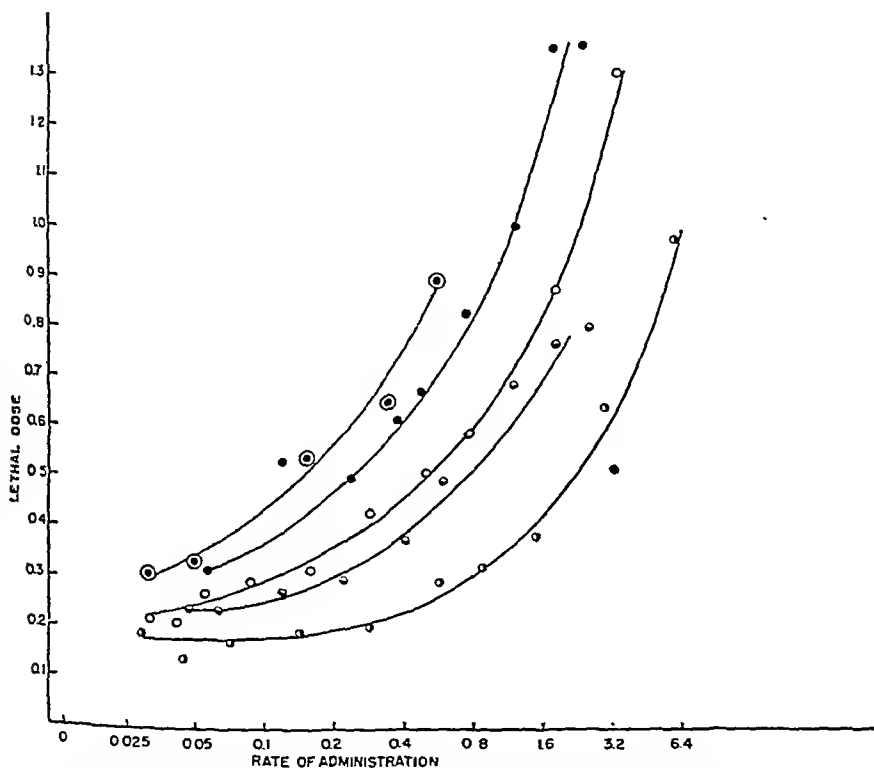


FIG. 1. Intact anesthetized cats. The relation of rate of administration of g-strophanthin, digoxin, oleandrin, and digitoxin to the lethal dose of these glycosides.

- g-strophanthin (Hildebrandt (13))
- digoxin
- oleandrin
- digitoxin (Hildebrandt (13))
- ⊙ digitoxin

Each point represents from 3-8 individual experiments.

digoxin. Oleandrin and digoxin have about the same activity. The value for the M.L.D. of our sample of digitoxin is somewhat higher than that obtained by previous investigators. We are unable to explain this discrepancy.

DISCUSSION. In the cat a reduction of the rate of administration of any of the cardiac glycosides studied, and the corresponding prolongation of the experimental time, invariably resulted in a decrease in the lethal dose until a definite value was reached which could not be lowered by further decrease in rate of

administration. The experimental times used have been extended to periods between 360 and 550 minutes. It is conceivable that longer times with cor-

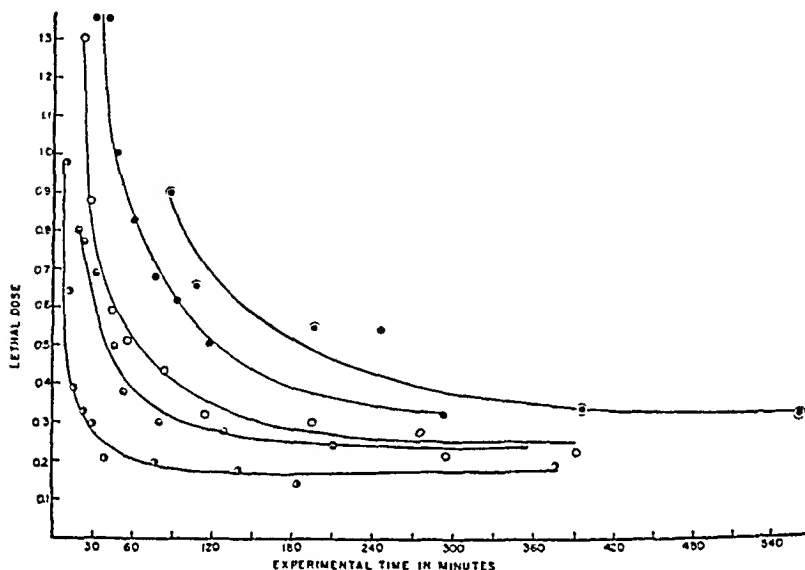


Fig. 2. Intact anesthetized cat. The relation of experimental time to the lethal dose of g-strophanthin, digoxin, oleandrin, and digitoxin.  
 ○—○ g-strophanthin (Hildebrandt (13))  
 ●—● digoxin  
 ○—○ oleandrin  
 ●—● digitoxin (Hildebrandt (13))  
 ○—○ digitoxin  
 Each point represents from 3-8 individual experiments.

TABLE 4

*Minimal lethal dose, optimal rate of administration and optimal experimental time of some cardiac glycosides*

	M.L.D.		OPTIMAL RATE		OPTIMAL TIME
	Micromols/ Kgm.	Mgm /Kgm.	Micromols/ Kgm /Hour	Mgm /Kgm / Hour	
g-Strophanthin . .	0.154	0.090	0.074	0.043	120-150
Oleandrin . . . .	0.208	0.120	0.052	0.030	270-300
Digoxin . . . .	0.230	0.180	0.055	0.043	240-270
Digitoxin . . . .	0.326	0.240	0.046	0.035	390-420

respondingly lower rates of administration might result in obtaining a lethal dose higher than the M.L.D. for the reasons discussed below. Such an increase in lethal dose has been described for tincture of digitalis in cats (11) and for certain glycosides in the guinea pig (20).

The M.L.D. as determined in this paper must have been influenced by factors of elimination. (Elimination is here used to include fixation and destruction of the active material by tissues, its excretion, and any shift of glycoside from the heart to extracardial tissue.) However the elimination rate in the cat for the glycosides studied is so slow (21, 22) that it is doubtful whether elimination has modified the M.L.D. to any appreciable extent. Lendle (21, 22) has made some measurements of the rate of elimination of g-strophanthin and of digitoxin in the cat. His method consisted of administering 80 per cent of the lethal dose of a glycoside and then determining the highest rate of administration of that glycoside which, over a period of 8 to 10 hours, would not kill the animal. This rate of administration was considered to be equal to the rate of elimination. This method will only give a rough approximation of the rate of elimination, since Lendle did not consider the fact that the amount of glycoside in the system probably plays an important role in determining the rate of elimination. Lendle's value obtained for g-strophanthin was 6 micrograms per kgm per hour. As the optimal experimental time for the determination of the M.L.D. of g-strophanthin is about 2 hours, the observed value of the M.L.D. (0.09 mgm per kgm) would have to be reduced by about 15 per cent to correct for elimination. This reduction is probably still within the error of the method of determination of the M.L.D. In the case of digitoxin the elimination rate found by Lendle was 3 micrograms per kgm per hour and thus the M.L.D. is influenced even to a lesser extent than that of g-strophanthin. No quantitative information on the rate of elimination of digoxin and oleandrin is available, but the values probably lie between those of g-strophanthin and digitoxin.

The lowering of the lethal dose due to a reduction in the rate of administration of the glycosides studied is not a phenomenon peculiar to the cat. Similar results have been obtained in the guinea pig (12) and in the dog (23). The reason for the reduction in lethal dose has not been determined with certainty. In the heart-lung preparation of the cat (24) and the dog (15) it has been shown that the heart binds a definite quantity of g-strophanthin regardless of the concentration of the glycoside in the blood. If this holds true for the intact animal the reduction in the lethal dose on reducing the rate of administration can not be explained on the basis that a smaller amount of glycoside is needed to kill the heart. In our opinion, the most important factor involved in the reduction of the lethal dose is the binding of cardiac glycosides by body tissues such as the liver, kidneys, and striated muscle. Weese (25) and Rothlin (26) have put forward evidence that these organs bind appreciable quantities of cardiac glycosides. Kiese, Gummel, and Garan (27) have studied the influence of rate of administration on the binding of g-strophanthin to liver tissue, using for this purpose the heart-lung-liver preparation of the dog. These investigators could show that with a reduction of the rate of administration the liver binds proportionally smaller quantities of this glycoside. They did not, however, consider the fact that with a reduction in the rate of administration the lethal dose for the heart-lung preparation decreases. Their data, therefore, have to be corrected. It is possible to utilize for this purpose the curve relating ex-

perimental time to lethal dose determined in the dog heart-lung preparation (15). The corrected values so obtained indicate that the reduction in the rate of administration markedly reduces the amount of g-strophanthin bound by the liver. It is quite conceivable that a similar process operates in the intact animal. The final solution of this problem will have to await the development of methods for the quantitative determination of cardiac glycosides in cardiac and extracardiac tissues.

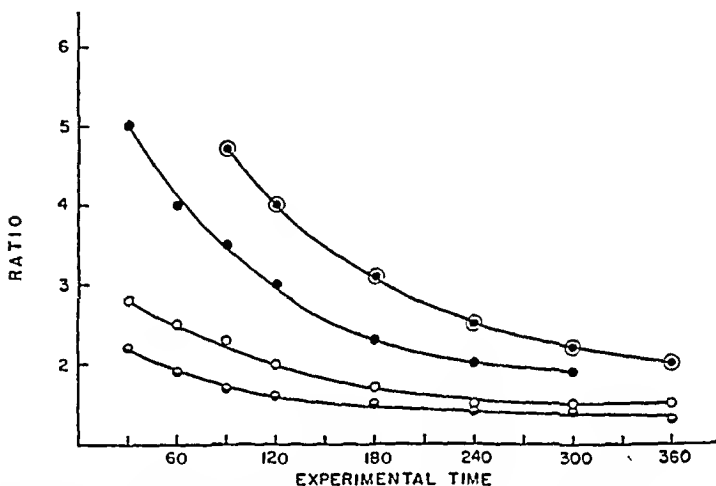


FIG. 3. The influence of experimental time on relative activity of a number of cardiac glycosides.

- lethal dose of digoxin
- lethal dose of g-strophanthin (Hildebrandt (13))
- lethal dose of oleandrin
- lethal dose of g-strophanthin (Hilderbrandt (13))
- lethal dose of digitoxin (Hildebrandt (13))
- lethal dose of g-strophanthin (Hildebrandt (13))
- lethal dose of digitoxin
- lethal dose of g-strophanthin (Hildebrandt (13))

In all the official cat methods for standardization of cardiac glycoside-containing material the experimental time is limited. Thus in the Magnus modification of the original Hatcher method the time limit is 35-60 minutes, while the U.S.P. XII adopted a time range of 65-90 minutes. That it is not sound to set such a time range is obvious from the curves in figure 2 relating experimental time to lethal dose.

Similarly, in an attempt to establish the relative potency of individual pure glycosides, it is unsound merely to relate the lethal dose of such glycosides to that of a reference standard, for instance, g-strophanthin. To illustrate this, the ratios of activity have been calculated at arbitrary experimental times by dividing the lethal dose of digitoxin, digoxin, and oleandrin, respectively, by the

lethal dose of g-strophanthin determined at the corresponding experimental time. A systematic variation in the ratio of potency for each glycoside becomes obvious if the ratio is plotted against experimental time as in figure 3. Unless the values of the M.L.D. are used, one can not obtain a valid concept of relative activity of cardiac glycosides even with the use of an appropriate reference standard.

White and Salter (28) have determined the molar ratio of activity of digitoxin to g-strophanthin in the failing papillary muscle of the cat and found a value of 2.2. The ratio of the M.L.D. of digitoxin to g-strophanthin as determined in the intact cat is 2.1. This agreement of the ratios of activity may be fortuitous. It would be of interest to see whether such agreement can be obtained with other glycosides.

Experiments to determine the ratio of oral to intravenous lethal dose have been based on values of the intravenous lethal dose determined by some modification of the Hatcher-Magnus method. Such experiments have been performed

TABLE 5  
*Comparison of Hatcher dose, intravenous minimal lethal dose and oral minimal lethal dose of some cardiac glycosides*

	HATCHER DOSE	M.L.D. (INTRAVENOUS)	M.L.D. (ORAL)	RATIO ORAL M.L.D. HATCHER DOSE	RATIO ORAL M.L.D. INTRAVENOUS M.L.D.
g-Strophanthin.....	0.1 (32)	0.09 (13)	2.2 (31)	22	24
Oleandrin .....	0.24 (32)	0.12	0.12 (29, 30)	0.5	1.0
Digitoxin.....	0.42 (32)	0.20-0.24	0.2 (29, 30)	0.5	1.0-1.2

by Fromherz and Welsch (29), van Esveld (30), and Svcc (31). The results show that by the oral route digitoxin and oleandrin are more potent than by the intravenous route of administration. In the experiments where the glycosides were given orally the experimental times were often over 5 hours. From what has been shown above, it would be more reasonable to compare the oral lethal dose to the intravenous M.L.D. This has been done in table 5 in which our own data and those of Fromherz and Welsch, van Esveld, Svcc, and Hildebrandt have been compiled. The data in this table show that the ratio of oral to intravenous M.L.D. is about 1 for digitoxin and oleandrin and about 23 for g-strophanthin. This is probably due to the better absorption of digitoxin and oleandrin as compared with g-strophanthin.

#### SUMMARY

The influence of rate of administration upon the lethal dose of digoxin, oleandrin, and digitoxin has been studied in intact cats. With high rates of administration the lethal dose is high. As the rate of administration is decreased the lethal dose falls until, over a wide range of very slow rates of administration, no further reduction in lethal dose occurs. This smallest lethal dose we refer to in this paper as the minimal lethal dose.

For each glycoside there is a characteristic maximal rate of administration (optimal rate of administration) at which the minimal lethal dose is determined (see table 4).

The methods in general use for determining relative activity of cardiac glycosides do not give the same ratios of activity as the values obtained when the minimal lethal dose is used. The use of the minimal lethal dose for comparison constitutes a reliable basis for relative potency.

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# RESPONSE OF PENICILLIN-RESISTANT STRAINS OF STAPHYLOCOCCUS AUREUS TO EXTRACTS OF BEEF BRAIN\*

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During the course of study of the effects of penicillin, sulfonamides and erythricin in various bacterial and parasitic diseases, it has been found that a favorable response was not always obtained in patients with staphylococcal infections (1-12).

In these laboratories, experiments to determine the effects of natural cellular substances on the growth of micro-organisms have been conducted for several years (13-16). An 80% alcohol-precipitated extract of beef brain tissue has been found to convert the yellow S-form of *Staphylococcus aureus* to the white R configuration, with concomitant stimulation of the growth of the latter form. Morphologic studies and experiments *in vivo* in mice have shown this white organism to be avirulent (14). The therapeutic and prophylactic effectiveness of brain tissue against *Staphylococcus aureus* infections induced subcutaneously, intraperitoneally, and intravenously has been established (14, 15). Later tests indicated that the extract is superior to penicillin in the dosages used for *Staphylococcus aureus* infections in mice (16).

On the basis of these findings, the present series of experiments was undertaken to determine the effect of brain extract on penicillin-resistant strains of *Staphylococcus aureus*. These studies included: (a) a comparison of the therapeutic and prophylactic activity of brain extract and penicillin on subcutaneous infections induced by 24 pathogenic penicillin-resistant strains of *Staphylococcus aureus*; and (b) a determination of the ability of these organisms to become insensitive to brain extract as shown *in vitro* by turbidity observations and *in vivo* using mice.

**EXPERIMENTAL.** The brain extract, made according to the procedure previously reported (13, 14), was prepared from ground beef brain which was extracted with an equal volume of water by alternate freezing and thawing over a period of 24 hours, the solid material removed by centrifugation, discarded and the proteins precipitated from the solution by bringing it up to a final concentration of 80% alcohol. The precipitated proteins were removed, the filtrate concentrated *in vacuo*, and further concentrated over a steam bath in open dishes until the content was 100 to 200 mgm. of solids per ml. Sterilization was accomplished by Seitz filtration. The various batches of extract were checked for effectiveness against a virulent strain of *Staphylococcus aureus* (ATCC 152) which responded to penicillin therapy (16). The penicillin was the commercial sodium salt obtained from Abbott Laboratories, Schenley Laboratories, and the Cheplin Laboratories.

The 24 penicillin-resistant organisms had been isolated from patients suffering from staphylococcal infections of various natures (chronic and acute osteomyelitis, empyema,

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axillary and retrobulbar abscesses, and soft skin infections) in which penicillin therapy had been reported unsuccessful.<sup>1</sup> In some instances the organisms had manifested a natural resistance to penicillin; in others, the organisms had apparently become resistant as a result of penicillin therapy since staphylococci isolated from the same lesions previous to treatment had been sensitive to penicillin. All strains were maintained on blood agar slants until time of use.

Rockland Farm albino mice from 3 to 6 months of age were used for the *in vivo* tests.

**Prophylactic Series. Control Animals.** For each of the 24 strains of *Staphylococcus aureus* there was a control group of 6 infected and untreated animals. Saline suspensions of staphylococci were prepared with 1 loopful of a 24-hour nutrient broth culture of the organism per ml. of saline. A uniform inoculum of 0.5 ml. was maintained for all strains and injections were made subcutaneously in the ventral abdominal region.

**Experimental Animals.** An initial subcutaneous dose of 1000 U. of the sodium salt of penicillin in 0.1 ml. of saline was given in the ventral abdominal region of mice in 24 groups of 6 animals each, 2 to 3 hours prior to subcutaneous infection with the staphylococci. Likewise, 50 mgm. of brain extract was given subcutaneously to mice in each of 24 groups of 6 animals each 2 to 3 hours prior to infection. The infecting dose of organisms was the same as that used for the control animals and daily treatment with 1000 U. of penicillin<sup>2</sup> and with 50 mgm. of brain extract was given until death of the animal or until the lesions were healed as gaged by the dropping off of the scab leaving smooth new skin beneath.

**Therapeutic Series.** Each mouse in this series received subcutaneously in the ventral abdominal region 0.5 ml. of a saline suspension of 1 loopful of a 24-hour culture of the organisms per ml. The animals were divided into control and experimental groups within 24 to 48 hours according to the degree of pathogenicity manifested. Animals showing similar degrees of reaction were distributed between the control and two experimental groups. For each of the 24 strains of staphylococci, 18 animals were used, 6 serving as infected untreated controls, 6 receiving penicillin, and 6 brain extract.

**Control Animals.** Mice in the control groups were injected each day with 0.1 ml. of saline, a volume equivalent to that in which the penicillin was given.

**Experimental Animals.** Subcutaneous treatment with 1000 U. of penicillin and 50 mgm. of brain extract was given daily, as soon as the animals were divided into experimental groups, and the treatment was continued until death of the animal or until the lesion healed completely.

**RESULTS. Prophylactic Series (Table 1).** In the control animals of the prophylactic series, the mortality ranged from 33½% with strain JB 9342 to 100% with 9 strains. Mortality for the total 144 control animals was 80% and occurred between the 1st and 24th days, half being dead on the 5th day (Fig. 1). Purulent lesions developed within 2 to 5 days and required 13 to 24 days (average, 19 days)

<sup>1</sup> We are indebted to Doctor C. H. Rammelkamp and Miss Marjorie Jewell of Evans Memorial Hospital, Boston; to Doctor W. W. Spink of the University of Minnesota; and to Doctor A. Bondi of Temple University, Philadelphia, for providing us with the 24 penicillin-resistant strains of *Staphylococcus aureus*. Those obtained from Evans Memorial Hospital were: JR 8742, JB 9342, JB 81142, AP 10242, AP 10742, HH 11242, Ram. 10342, Ram. 92442, Ram. 91342, Ram. 10842, Ram. 87, Rosen 41, Merendale 515, Larabee 423, Nicolazzo 1124, Walbourne 411; those from Temple University were Bondi 446 and Bondi 161 and from the University of Minnesota, Rosen II CPF, Rosen III PF, Rosen I, Long III PF, Bernardo IIA, Bernardo II.

<sup>2</sup> From the results of preliminary experiments with the penicillin-resistant strains of organisms in which the penicillin was given at 6 hourly intervals and once daily, the single daily injection appeared to be as effective as the multiple daily injections. For this reason, the single daily dose of the penicillin was used.

for complete healing in the survivors. Abscess formation was followed by an extensive sloughing and the appearance of signs of listlessness, shaggy hair, cyanosis, and paralysis of the limbs.

Of the 144 animals treated with penicillin, 80% died within the first 17 experimental days, more than half being dead on the 5th day. In the individual groups, the mortality ranged from 33½% with 4 strains of organisms to 100% with 14 of the penicillin-resistant strains. All of the survivors in these groups of animals developed purulent lesions averaging 18 days for healing with a range of 8 to 24 days.

TABLE 1

*Comparative response of mice infected with penicillin-resistant strains (24) of Staphylococcus aureus to prophylactic and therapeutic administration of penicillin and brain extract*

NO. ANIMALS	TREATMENT	% MORTALITY	HEALING TIME SURVIVORS
Prophylactic Series			
			days
144	Control	80.0	19 (13-24)
144	Penicillin	80.0	18 (8-24)
144	Brain Extract	0.6	11 (4-24)
Therapeutic Series			
144	Control	84.0	18 (7-25)
144	Penicillin	95.0	22 (16-26)
144	Brain Extract	1.4	7 (3-17)

18 mice used for each penicillin-resistant strain of organisms. 6 control and 6 in each of the treated groups.

Inoculant 0.5 ml. of a 24-hour nutrient broth culture, 1 loopful per ml.

In the therapeutic series, treatment consisted of 50 mgm. brain extract per day, 1000 U. sodium salt of penicillin, daily, while the controls received 0.3 ml. saline daily.

In the prophylactic series both initial and subsequent doses were 50 mgm. of brain extract and 1000 U. penicillin, daily.

Of the 144 animals receiving brain extract, 1 died of an injury, none died from the infection. Dry, scaly, non-suppurating lesions accompanied by severe sloughing in some cases developed within 2 to 5 days in only 86% of the group and these healed within 4 to 24 days (11 days average). There were no signs of toxic symptoms or paralysis among the brain extract treated animals.

*Therapeutic Series (Table 1).* In the 144 saline-injected control animals, mortality ranged from 66½% with 5 strains to 100% in 6 strains. Mortality for the entire group was 84%, more than half being dead on the 3d experimental day (Fig. 1). In the survivors, the purulent lesions required 7 to 25 days (average, 18) for complete healing.

Of the 144 animals receiving penicillin, the total mortality was 95% ranging from 83½% in 7 strains to 100% in 17 strains. More than one-half of the animals

were dead on the 3d experimental day. The purulent lesions in the survivors required 16-26 days (average, 22) for complete healing.

Of the 144 animals treated with brain extract, 2 died; 1 (HH 11242) on the 2d and 1 (Bondi 161) on the 3d experimental day. The healing time of the lesions in the 142 survivors ranged from 3 to 17 days with an average of 8 days.

**FAILURE OF PENICILLIN-RESISTANT STRAINS OF STAPHYLOCOCCUS AUREUS TO BECOME RESISTANT TO BRAIN EXTRACT, IN VITRO AND IN VIVO.** *Procedure. In vitro.* For each of the 24 penicillin-resistant strains of staphylococci, 3 tubes were prepared containing 10 ml. of nutrient broth and 0.2 ml. veal infusion. One of

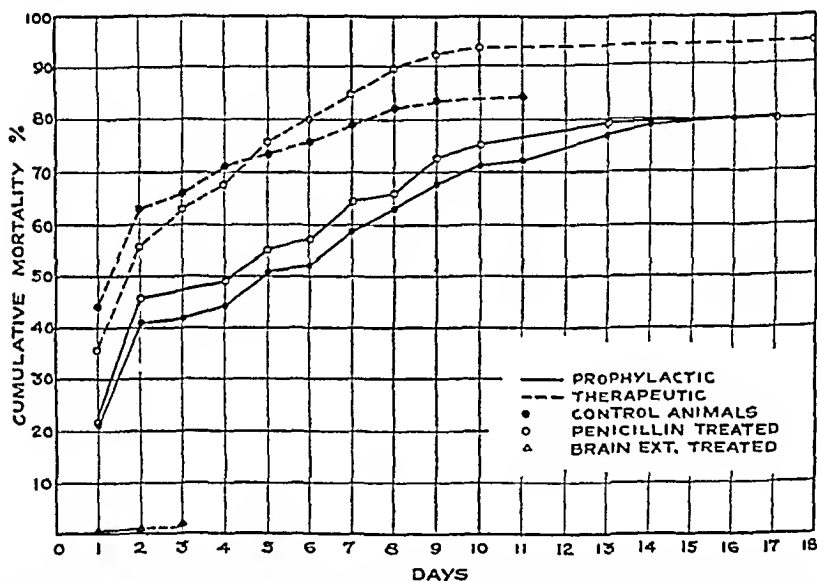


FIG. 1. Cumulative mortality in a prophylactic (432 mice) and a therapeutic (432 mice) series of animals testing the effectiveness of brain extract against 24 penicillin-resistant strains of *Staphylococcus aureus*.

these served as a control. To 1 was added 57.1 U. of penicillin in the form of a freshly prepared 0.85% saline solution of the sodium salt of penicillin, containing 20 U. per ml. To the 3d tube 100 mgm. of brain extract (100 mgm. per ml.) was added. Each tube was inoculated with a standard loopful of a 24-hour nutrient broth culture of the organism and incubated at 37°C. for 24 hours. Direct transfers were made every 24 hours (except for Sundays and holidays) to an identical series of 3 culture tubes. Twelve of the strains of organisms were thus treated for 37 days and the remaining 12 strains for 74 days. Visual determination of the turbidity was made each day.

*In vivo.* For each of the 24 penicillin-resistant strains of *Staphylococcus aureus*, 3 test tubes containing nutrient broth media were inoculated with a standard

loopful of a 24-hour culture of the organism. One of the tubes served as a control. To 1 experimental tube 57.1 U. of penicillin in the form of the sodium salt in saline (20 U. per ml.) was added, and to the other, brain extract (100 mgm. per ml.) and the tubes were incubated at 37°C. Transfers were made every 3 days. At the expiration of a 12-hour, a 15-day, and a 30-day incubation period, the potency of the cultures was determined by tests in animals. Rockland Farm albino mice averaging 3 to 6 months of age were inoculated subcutaneously with the organisms in the ventral abdominal region. The inoculum dosage for each

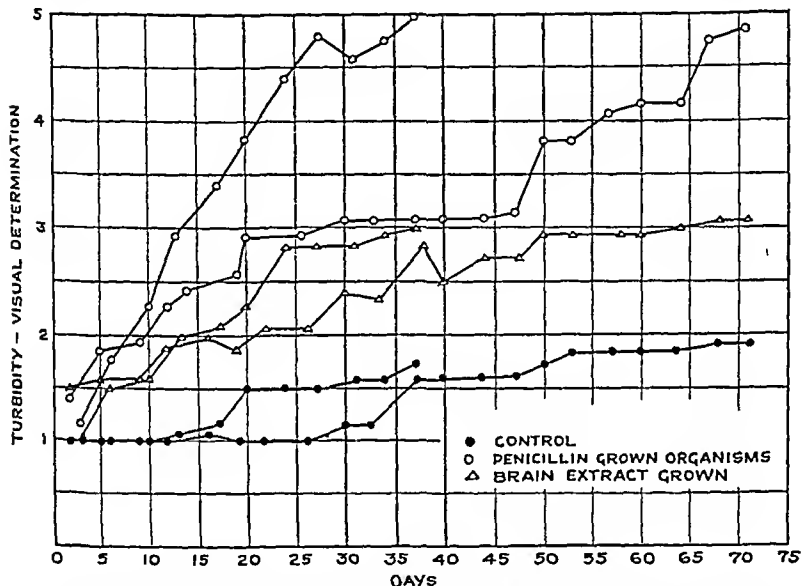


Fig. 2. Average turbidity in two series of penicillin-resistant strains of *Staphylococcus aureus*, 12 per series, cultured for 37 and 74 days, respectively, in the presence of penicillin and of brain extract.

Although it would appear from the graph that the organisms were stimulated only slightly less with brain extract than with penicillin, this is not an indication of the development of resistance in the case of the brain extract for its action is one of stimulation of growth with a concomitant conversion of the virulent organism to an avirulent form.

strain was 0.5 ml. of a 24-hour nutrient broth culture. Observations were made on the mortality and healing time of the lesions.

**Results. In vitro.** In Fig. 2, the turbidity readings selected for graphing were those for Tuesdays and Saturdays. Each point on the graph represents an average of the readings for the 12 strains on the specific days. The 2d series of 12 organisms (37-day experiment) was begun 2.5 weeks after the completion of the 1st series of 12 (74-day experiment). It is apparent that the over-all turbidity readings of the 2d series are above those of the 1st series and it is felt that this apparent increase was due to the personal factor in visual determination.

Within each series, however, the comparative control and experimental values are probably consistent.

Although it would appear from the graph that there is only a slight difference in the amount of stimulation of the growth of the microorganism by brain extract, as compared with penicillin, the action of these two materials is entirely different. Penicillin, when effective, acts by inhibiting growth while the effectiveness of brain extract depends on the conversion of the yellow organism to a white variant with concomitant stimulation of growth (14, 15). It is apparent,

TABLE 2

*Comparative mortality in mice infected with penicillin-resistant strains (24) of Staphylococcus aureus cultured 12 hours, 15 and 30 days in a control media and in media containing penicillin and brain extract*

NO. ANIMALS	CULTURE	% MORTALITY	HEALING TIME SURVIVORS
12-hour Cultures			
144	Control	82.0	days
144	Penicillin	95.0	*
144	Brain Extract	2.8	*
15-day Cultures			
120	Control	54.0	19 (10-32)
120	Penicillin	90.0	22 (9-30)
120	Brain Extract	3.3	11 (4-23)
30-day Cultures			
120	Control	60.0	17 (4-29)
120	Penicillin	100.0	—
120	Brain Extract	0.0	7 (4-16)

\* No healing data recorded for test animals of 12-hour cultures.

18 mice were used for each strain of the penicillin-resistant organisms cultured for 12 hours; 15 mice for each strain cultured for 15 and 30 days; the animals were divided into 3 groups of equal number.

Inoculant 0.5 ml., 1 loopful per ml.

therefore, that throughout the experimental periods (37 and 74 days) the organisms remained sensitive to the action of the brain extract, while in the presence of penicillin they retained their resistance to this antibiotic.

*In vivo* (Table 2, Fig. 3). *Twelve-hour cultures* (432 animals). Total mortality in the control group was 82% and ranged from 33½% for 1 strain (Rosen I) to 100% for 12 strains; 71% died within 48 hours. For the animals receiving organisms from the penicillin-containing culture, the total mortality was 95% (range 66½% in 1 strain to 100% in 17 strains); 85% were dead within 2 days. Total mortality for the animals receiving organisms grown for 12 hours in cultures containing brain extract was 2.8%. One died on the 1st day (Bernardo IIA), 2 on the 3d (Nicolazzo 1124) and the last on the 4th day (Ram. 92442). No ob-

servations were made on the time required for healing of the lesions in the survivors of this group. One animal of the group receiving Rosen II CPF organisms had a lesion in which there was suppuration for 2 days.

*Fifteen-day cultures (360 animals).* The total mortality in the control series was 54% (range of 20% in Rosen 41 to 80% in 4 strains). The highest mortality occurred on the 3d day by which time 27% of the control animals were dead. The purulent lesions in the survivors required an average of 19 days for healing (range 10-32 days).

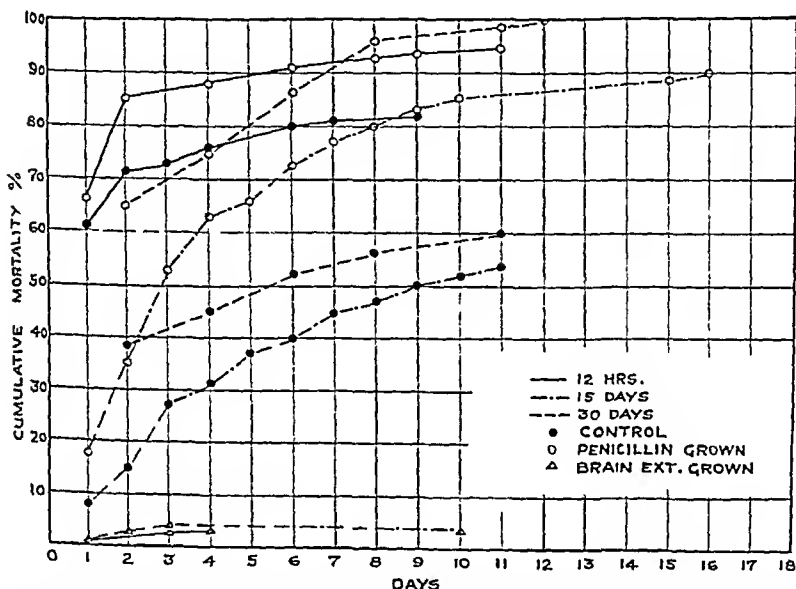


Fig. 3. Cumulative mortality in mice following infection with penicillin-resistant *Staphylococcus aureus* organisms cultured for 12 hours (432 mice), 15 days (360 mice) and 30 days (360 mice) in the presence of penicillin and of brain extract.

The total mortality for the animals receiving organisms cultured for 15 days in the presence of penicillin was 90%, with a range of 60% for 4 strains to 100% for 16 strains; 53% were dead by the end of the 3d day. The lesions were purulent and required 22 days (range 9-30) for complete healing.

Of the 120 animals receiving organisms cultured for 15 days with brain extract incorporated in the media the total mortality was 3.3%, 1 animal dying on each of the 1st 3 days (Ram. 10842, Bondi 161, and Bernardo IIA), the 4th on the 10th day (Merendale 515). On the 1st day all of the animals receiving Bondi 161 had slightly suppurating lesions. One of these died, and suppuration disappeared in 2 by the 2d day and in the remaining 2 by the 4th day. One animal of the group receiving Rosen II CPF had a slightly suppurative lesion on days 2 and 3. The healing time for the lesions was 11 days (range 4-23 days) in the animals of this group.

*Thirty-day cultures (360 animals).* Total mortality among the 120 control mice was 60 per cent, ranging from 40% for 5 strains to 80% for 5 strains; 45% were dead by the 4th day. The average healing time for the purulent lesions in the survivors was 17 days with a range of 4 to 29 days.

Total mortality for the 120 animals receiving organisms grown for 30 days in the presence of penicillin was 100% within 12 days; 76% were dead by the 4th day.

There was no mortality among the 120 animals receiving organisms grown for 30 days in the presence of brain extract. The lesions, which developed in but 84% of the animals, were non-suppurating and required an average of 7 days for complete healing (4-16 days range). They were healed by the 11th day in 17 of the 24 groups of animals.

#### SUMMARY

Infections in mice with a series of 24 proven penicillin-resistant strains of *Staphylococcus aureus* responded to prophylactic and therapeutic administration of 80% alcohol-precipitated beef brain extract. The turbidity studies on penicillin-resistant organisms cultured in the continuous presence of brain extract for periods of 74 and 37 days showed an increased growth as well as a conversion of the yellow S organism to a white R form thus indicating that the organisms were sensitive to the extract throughout the experimental period. Similar studies with organisms cultured in the presence of penicillin demonstrated that the resistance to penicillin was maintained as evidenced by increased turbidity. In vivo tests, after 12 hours, 15 and 30 days, of culture under these experimental conditions demonstrated that the organisms became avirulent in the presence of the brain extract and that this sensitivity to the extract was maintained throughout the experimental period.

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# THE INFLUENCE OF DIET ON THE SUSCEPTIBILITY OF RATS TO ALPHA-NAPHTHYLTHIOUREA

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Several previous investigations have indicated that the acute toxicity of thiourea is influenced by dietary substances. Thus, Dieke and Richter (1) found that a low protein diet increased the susceptibility of rats to thiourea while Landgrebe and Morgan (2) noted that rats maintained on an oatmeal diet became extremely sensitive to thiourea. Griesbach et al (3) observed that the injection of iodine afforded protection against acute thiourea poisoning if the iodide was administered prior to thiourea.

The new rodenticide, alpha-naphthylthiourea (ANTU), is similar in many of its chemical and physiological properties to the parent compound, thiourea. One of the most marked characteristics of ANTU is a wide species difference and a less prominent strain difference in susceptibility (4). In view of certain similarities between ANTU and thiourea and because of the marked species differences in susceptibility to the rodenticide it was of interest to ascertain the influence of dietary factors on the toxicity of ANTU, since diet might be a contributing factor in the species differences. In the present study the influence of variations in dietary fat, carbohydrate, protein, and cystine on the toxicity of ANTU was examined.

Preliminary studies in this laboratory (5) have shown that the addition of iodine to the diet or drinking water affords considerable protection to rats against ANTU provided that the iodine is administered prior to ANTU. Further information on the protective action of iodine against acute ANTU poisoning is included in this communication.

**METHODS AND MATERIALS.** Two to three month old male and female Sprague-Dawley rats were used throughout these experiments. The animals were maintained in individual cages in a room in which the temperature was regulated between 67° and 75°F. The animals were given distilled water and diets of known composition were fed *ad libitum*. Food intake was recorded every second day and the animals were weighed at 5-day intervals. From these data the average food intake and average change in weight were obtained during the feeding period.

For measuring the toxicity of ANTU the rodenticide was dissolved in anhydrous propylene glycol and injected intraperitoneally in volumes not exceeding 0.5 ml. per rat. The LD 50 values for ANTU were calculated from the experimental mortality data by the method

<sup>1</sup> The experimental data in this paper are taken from a thesis submitted in *absentia* in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biochemistry in the Graduate School of the University of Illinois.

<sup>2</sup> This work was carried out under contract with the Medical Division of the Chemical Warfare Service.



of Bliss (6). The fiducial limits ( $p = 0.003$ ) of the LD 50 were calculated by adding to and subtracting from the log of the LD 50 a quantity equal to three times the log of the standard error of the LD 50.

DIETS. As control diets Purina Chow and a purified diet containing casein (Merk edible) 18%, dextrin 50%, cerelese 15%, cellu flour 2%, salt (7) 3%, cod liver oil 4%, lard 4%, corn oil 4%, and a vitamin supplement were fed. The vitamin supplement, which was thoroughly mixed with 200 grams of casein before incorporation with the remainder of the diet, contained, per 100 grams of diet, thiamine hydrochloride 0.25 mgm., pyridoxine hydrochloride 0.25 mgm., nicotinic acid 0.25 mgm., riboflavin 0.5 mgm., choline chloride 125 mgm., and Wilson's 1-20 liver extract 625 mgm.

TABLE 1

*The composition of experimental diets in which variations were made in protein, carbohydrate, and fat*

DIET	DIET NUMBER	CASEIN % OF DIET	DEXTRIN % OF DIET	LARD % OF DIET
Control	1	18	50	4
High carbohydrate 12% protein	2	12	56	4
High fat 12% protein	3	12	37	23
40% protein	4	40	28	4
6% protein	5	6	62	4
0% protein	6	0	68	4

TABLE 2

*The composition of high cystine diets*

DIET	CONSTITUENT		
	Diet number	Cystine % of diet	Choline chloride % of diet
1% Cystine diet	7	1	0.125*
5% Cystine-0% choline diet	8	5	0
5% Cystine-0.6 choline diet	9	5	0.6

\* Quantity of choline chloride in normal control diet.

Five of the experimental diets were prepared by varying one of the three principal dietary components, carbohydrate, protein, and fat. The variations in fat were effected by changing the quantity of lard employed, in carbohydrate by altering the proportions of dextrin, and in protein by varying the quantity of casein. The variations in these dietary constituents are presented in table 1. In the diet which contained no casein the vitamins were first mixed with cerelese before incorporation into the remainder of the diet.

A group of three diets indicated in table 2 were prepared to ascertain the effect of high dietary cystine on the resistance of rats to ANTU. Cystine and choline chloride were added to the 18% casein control diet at the expense of dextrin.

To ascertain the effect of dietary iodine upon the susceptibility of rats to ANTU the diets indicated in table 3 were prepared. These diets were compounded by adding finely powdered potassium iodide to the control diet at the expense of dextrin. When iodine was supplied in drinking water it was added either as potassium iodide or Lugol's solution.

**EXPERIMENTAL.** *Effect of variation in carbohydrate, fat, and protein on the toxicity of ANTU.* The toxicity of ANTU to rats fed rations in which variations were made in carbohydrate, fat, and protein are presented in table 4. Each LD 50 was calculated from at least three doses at which the mortality was between 20% and 80%. These mortality data were obtained by measuring the toxicity of ANTU to two separate groups of 10 or more animals injected with ANTU at

TABLE 3  
*The composition of high iodine diets*

DIET NUMBER	IODINE NO. IODINE/G.	FORM OF IODINE USED	VEHICLE
10	2.5	Lugol's soln.	drinking water
11	2.5	KI	drinking water
12	5.7	KI	diet
13	7.6	KI	diet
14	2.1	KI	diet
15	0.9	KI	diet

TABLE 4  
*The effect of variations in protein, carbohydrate, and fat upon the resistance of rats to ANTU*

DIET AND DIET NUMBER	FEED- ING PERIOD	NO. OF ANI- MALS USED	LD 50 (CALC- ULATED)	FIDUCIAL LIMITS LD 50 (p-0.003)	AVE. WEIGHT GAIN/DAY		AVE. FOOD CONSUMED PER DAY		AVG. WT. OF ANIMAL AT TIME OF IN- JECTION
					male	female	male	female	
Purina Laboratory Chow control	days		mgm./kgm.						
	14	90	6.80	5.98-7.73					225
1	10	26	ca 6.3		3.0	1.4	16	12	220
1	30	22	ca 6.5		3.5	1.5	16	12	300
18% control (combined)		48	6.5	5.8-7.1					
4	30	35	6.6	5.5-8.0	3.7	—	16	—	284
3	30	24	ca 5.3		1.7	—	14	—	250
2	30	25	4.7		3.6	1.3	16	12	272
12% protein (2+3 combined)		49	5.9	5.6-6.2					
5	30	35	3.5	2.9-4.3	0.4	-0.6	12	12	222
6	30	29	3.8	3.0-4.7	-3.6	-3.0	7	7	125

different times of the year. No indications of seasonal variations in susceptibility were observed, however, under the conditions of these tests.

It is indicated in the results shown in table 4 that the LD 50 of ANTU for animals eating Purina Laboratory Chow and the synthetic control diet were not different. The length of the feeding period did not affect the resistance of animals fed the synthetic control diet and the values obtained for different feeding periods were therefore combined so that a more accurate calculation of the LD

50 could be made. The LD 50 for animals receiving Purina Chow was 6.8 mgm./kgm. as compared with 6.5 mgm./kgm. for animals eating the synthetic control diet.

It may be seen in table 4 that feeding a 40% casein diet did not materially affect the LD 50 of ANTU for rats. When two 12% protein diets were fed, one high in fat and the other high in carbohydrate, the resistance to ANTU appeared to be slightly lower but the decrease was not significant by the statistical method employed. The slight increase in susceptibility was related to the protein concentration, however, and not to variations in fat or carbohydrate since varying the quantity of either carbohydrate or fat in the diet had no effect upon the resistance of rats to ANTU. The mortality data obtained after feeding the two 12% protein diets but different amounts of carbohydrate and fat were, therefore, combined to give a more accurate calculation of the LD 50 of ANTU to rats receiving 12% protein.

TABLE 5

*The influence of high cystine diets upon the resistance of rats to ANTU*

DIET NO.	FEEDING PERIOD	NO. OF ANIMALS USED	LD 50 (CALCULATED)	FIDUCIAL LIMITS LD 50 ( $p = 0.003$ )	AVE. FOOD INTAKE	AVE. WT. GAIN	AVE. WT. OF ANIMALS AT TIME OF INJECTION
	Days		mgm./kgm.		g./day	g./day	
7	5	30	12.2	9.2-16.1	16.5	4.5	229
7	10	26	11.8	8.7-16.1	16.5	4.5	220
7	20	26	7.5	6.1-9.6	16.0	3.0	285
7	30	26	6.2	4.3-8.0	16.5	4.0	315
9	10	20	9.8	7.4-13.1	16.0	4.5	210
8	10	20	5.2	3.5-7.8	10.0	0.5	195

It will be noted that lowering the protein of the diet to levels which did not support growth (diets 5 and 6, table 1) resulted in a significant increase in susceptibility toward ANTU. The LD 50 for rats receiving both the 6% protein and 0% protein diets was about 3.5 mgm./kgm. with an upper fiducial limit ( $p = 0.003$ ) of 4.7 mgm./kgm. which is substantially below the lower fiducial limit (5.8 mgm./kgm.) of the control LD 50.

*Effect of dietary cystine on the toxicity of ANTU.* The influence of an increased quantity of dietary cystine on the susceptibility of rats to ANTU is shown by the data in table 5. It may be seen that the calculated LD 50 of ANTU for the groups of animals which had received the 1% cystine diet for five days was 12.3 mgm./kgm. with fiducial limits of 9.2 to 16.2 mgm./kgm. This LD 50 was significantly above the control value. After feeding 1% cystine for 10 days protection against ANTU was still observed, for an LD 50 of 11.8 mgm./kgm. was obtained. However, when this high cystine diet was fed for 20 days rats no longer showed increased resistance to ANTU as was indicated by an LD 50 of 7.5 mgm./kgm. of ANTU. After feeding the diet for 30 days the LD 50 for ANTU was 6.5 mgm./kgm. which is the same as the control LD 50.

It will be noted that the average weight gain and food consumption of animals receiving the 1% cystine was similar to that of the control animals which had received a normal diet.

When a 5% cystine-0.6% choline diet was fed some protection against ANTU was observed but it was no greater than that observed after feeding a 1% cystine diet. When 5% cystine was added to a diet containing no choline, added protection against ANTU was lost. When choline was added to the high cystine diet growth and food consumption was similar to that of the controls and some added resistance toward ANTU was observed, while animals receiving a high cystine diet which was deficient in choline grew at a retarded rate, showed decreased food consumption, and exhibited no added resistance toward ANTU.

*Effect of Iodine on the toxicity of ANTU.* Preliminary experiments in this laboratory (5) have indicated that iodine given either as Lugol's solution or as potassium iodide increased the resistance of rats to ANTU. Quantities of iodine from 20.8 to 44.8 mgm./kgm. of iodide given over a 1-2 day feeding period re-

TABLE 6

*The influence of high iodine diets upon the resistance of rats to ANTU*

DIET NO.	CONCN. OF IODINE MGM. IODINE/g.	FEEDING PERIOD	NO. OF ANIMALS USED	AVERAGE QUANTITY OF IODINE CONSUMED	LD-50 (CALCULATED)	FIDUCIAL LIMITS LD 50 (p = 0.003)
		days		g /kgm	mgm /kgm	
10	2.52	1	24	0.22 (0.12-0.45)	30	26-35
11	2.52	2	33	0.30 (0.16-0.53)	33	29-37
12	5.7	2	28	0.42 (0.28-0.59)	42	34-52
13	7.6	2	24	0.76 (0.49-1.14)	56	52-66
12	5.7	5	40	1.37 (0.65-1.76)	76	63-91
13	7.6	5	24	1.85 (0.91-2.46)	80	66-92
12	5.7	10	20	2.89 (2.14-4.02)	91	70-117
14	1.9	10	25	1.28 (0.88-1.75)	61	50-73
15	0.9	30	20	1.83 (1.64-2.06)	31	24-44

sulted in an increase in the LD 50 to values as high as 44 mgm./kgm., the amount of resistance being dependent upon the quantity of iodine ingested. The results of more extensive studies on the protective effect of iodide are summarized in table 6, in which the relationship between the quantity of iodine, the length of the feeding period, and the resistance acquired toward ANTU are shown.

The data in table 6 indicate that iodine given either in the drinking water or in the diet endows rats with marked resistance toward ANTU. When a diet containing 5.7 mgm. of iodine/gram was fed for two days the LD 50 of ANTU increased from a normal of 6.8 mgm./kgm. to 42 mgm./kgm. and after feeding this same diet for 5 days the LD 50 further increased to 76 mgm./kgm. An LD 50 of 91 mgm./kgm., which was a 13-fold increase over the value for control animals, was obtained when animals had received the ration for 10 days. It is seen from these experiments that the amount of protection afforded by a given amount of iodine in the diet increased with the length of the feeding period at least during the first 10 days.

The quantity of iodine ingested during a given period also influenced the amount of resistance acquired by rats as is shown in table 6. Whereas a diet containing 2.52 mgm. iodide per gram fed for two days increased the LD 50 to 33 mgm./kgm. feeding a diet containing 7.6 mgm. iodine per gram for 2 days provided greater protection to rats, the LD 50 being 56 mgm./kgm.

Lugol's solution and potassium iodide were of nearly equal effectiveness when provided at equal iodine concentrations in the drinking water. Bromides offered no protection when fed in the drinking water or the diet.

It will be noted that when a given dose of iodine was fed for 10 days it gave somewhat less protection than was obtained by feeding the same amount of iodine during a 5-day feeding period. Thus after a dose of 1.37 grams of iodine/kgm. provided by feeding rats a diet containing 5.7 mgm. iodine per gram of the diet for 5 days the LD 50 of ANTU was 76 mgm./kgm. When this same dose of iodine was given over a 10-day period by feeding a diet containing 2.1 mg. iodine per gram for 10 days the LD 50 was 61 mgm./kgm., which value is below the lower fiducial limit of the LD 50 (76 mgm./kgm.) obtained after feeding the same quantity of iodine during a 5-day period. This effect was more clearly shown by feeding 0.9 mg. iodine/gram of diet for 30 days which resulted in an average iodine intake of 1.83 grams/kgm. The LD 50 for 20 animals on this diet was 31 mgm./kgm. which is substantially lower than was obtained with an equivalent amount of iodine given over a 5-day period. It is apparent from these experiments that part of the protective effect of a given dose of iodine is lost when it is administered over a long period of time.

Growth rate was followed for one group of rats receiving the high iodine diet (diet 12) and the average weight increase was 2.7 grams per day as compared with 3.0-4.5 grams for the controls. Food consumption for those animals eating the iodine diet was slightly below that for the control animals.

*The influence of intravenous iodine on the toxicity of ANTU.* Since Roblin et al. (8) have shown that a direct reaction readily takes place between iodine and thiourea derivatives *in vitro* and DuBois and Erway (9) found that the inhibition of tyrosinase by ANTU could be prevented by the addition of small quantities of iodine to the test system it seemed possible that interaction of iodine and ANTU might result in detoxification of ANTU *in vivo*. If such were the case one might expect iodine administered intravenously immediately before or even after the administration of ANTU to exhibit a protective effect.

For these experiments Lugol's solution was diluted so that the iodine concentration was 126 mgm./cc. This solution was injected into the tail vein of rats and 2 minutes later the animals were given ANTU intraperitoneally. While only half of a group of control animals survived 50 mgm./kgm. of iodine given intravenously 25 mgm./kgm. of iodine was not lethal to any of the control animals. The LD 50 of ANTU for animals receiving 25 mgm./kgm. of iodine 2 minutes before the ANTU was 6.5 mgm./kgm. as compared with 6.8 mgm./kgm. for normal animals receiving no iodine. Since no protection was afforded by intravenous iodine given immediately before ANTU it is seen that the immediate concentration of iodine in the tissues had little effect upon the resistance of rats

toward ANTU. This appears to eliminate a direct reaction between ANTU and iodine as an explanation for the protective effect of iodide.

*Effect of high iodine diets on the susceptibility of thyroidectomized rats to ANTU.* Since iodine did not appear to exert its protective effect through a direct reaction with ANTU it was necessary to consider indirect mechanisms which might be involved in the protective effect. In this connection it was of interest to examine the effect of high iodine diets upon the resistance of thyroidectomized rats to ANTU. The rats were thyroidectomized at least two weeks prior to being used for these experiments.

The LD 50 of ANTU to a group of 21 thyroidectomized animals receiving a normal diet was 4.7 mgm./kgm. as compared with an LD 50 of 6.8 mgm./kgm. for normal rats fed an adequate diet. The LD 50 of ANTU to a group of 21 thyroidectomized animals eating a diet containing 5.7 mgm. of iodine per gram of diet for 5 days was 8.5 mgm./kgm. These animals consumed an average of 1.0 gram of iodine/kgm. of body weight during the 5-day feeding period whereas normal animals consumed 1.37 grams of iodine/kgm. during a similar period. Whereas 1.0 gm./kgm. of iodine produced little protection over a 5-day feeding period in thyroidectomized rats the LD 50 for control animals receiving the same quantity of iodine increased to about 60 mgm./kgm. From these experiments it may be concluded that iodine affords very little protection against ANTU to thyroidectomized rats indicating that the thyroid gland is involved in some manner in the protective action of iodine against acute poisoning by ANTU.

*Effect of desiccated thyroid on ANTU poisoning.* The absence of the protective effect of iodine on ANTU poisoning in thyroidectomized animals suggested testing the effect of desiccated thyroid added to the diet on the toxicity of ANTU in order to ascertain whether iodide exerted its protection by increasing the quantity of thyroxin or diiodotyrosine in rat tissues. A diet containing 12.5 mgm. of desiccated thyroid per gram was fed for two days. This feeding period was chosen because inorganic iodides were effective in affording protection against ANTU if given 6 hours prior to ANTU. Thus, if inorganic iodides were converted to di-iodotyrosine or thyroxin such a conversion must have taken place within 6 hours. A group of 10 rats consumed an average of 15.3 grams of food per rat per day which is comparable to normal food consumption and they lost an average of 10.1 grams per rat per day which indicated that the desiccated thyroid had some effect on metabolism. None of these animals survived 10 mgm./kgm. of ANTU indicating that desiccated thyroid exerts no protective action against ANTU in the doses given in this experiment.

If the protective action of iodide involved conversion to di-iodotyrosine or thyroxin then the latter substances should be at least as effective as inorganic iodide in offering protection against ANTU. However, the injection of 16.9 mgm./kgm. of di-iodotyrosine (containing 10 mgm./kgm. of iodine) immediately before ANTU or 24 hours prior to ANTU afforded no protection while a similar quantity of inorganic iodide protected some of the animals against the lethal effects of 10 mgm./kgm. of ANTU.

DISCUSSION. The results of these studies indicate that variations in dietary

carbohydrate, fat, and protein, the three main constituents of the diet, had no effect on the susceptibility of rats to ANTU except when low protein diets, inadequate for growth, were fed. Lowering the protein content of the diet to 6% or to 0% resulted in a significant reduction in resistance to ANTU. These results with ANTU are in accord with observations by Dicke and Richter (1) who found that a low protein diet increased the susceptibility of rats to thiourea although those investigators found a much more pronounced effect on susceptibility to thiourea than was noted in our experiments with ANTU. From the experiments reported here it is apparent that the great difference in susceptibility of omnivorous and herbivorous animals to ANTU cannot be ascribed directly to differences in protein, carbohydrate, or fat content of their immediate diet.

It was found that certain other constituents of the diet increased the resistance of animals to ANTU. A 1% cystine diet fed for 5 days nearly doubled the resistance to ANTU and the same amount of resistance was observed when the diet was fed for 10 days. It was interesting that the protection by cystine was no longer observed when the diet was fed for 30 days and that increasing the amount of cystine from 1 to 5% did not increase the amount of protection. It appears that the protection by cystine was not directly related to the absolute quantity of cystine eaten in any given feeding period and cystine was ineffective in protecting animals against ANTU unless choline was also added in adequate amounts. The mechanism underlying this transient protection by cystine was not examined in these studies but it seems possible that it might involve an interference by cystine with thyroid function.

Of the dietary constituents examined iodide provided the greatest amount of protection against ANTU for it was possible to produce a 13-fold increase in the resistance of rats to ANTU by feeding a diet high in iodine for 10 days. That protection could not be obtained by the administration of iodine immediately prior to or after ANTU seems to eliminate direct interaction of ANTU and iodine as an explanation for the protective action. The inability of iodine to protect thyroidectomized rats indicates that the protective action of iodide is mediated through the thyroid gland. This suggested that the protective action of iodide might depend on its conversion to di-iodotyrosine or thyroxine. Such a conversion would explain the necessity of administering the iodide at least 6 hours prior to ANTU. However, no evidence in support of such an explanation was obtained in the experiments presented here. Neither di-iodotyrosine nor thyroid extract offered any protective effect in the doses administered. Studies on the distribution of iodine in the tissues of ANTU-poisoned animals as well as the tissues from animals fed large amounts of iodine seem worthwhile in attempting to elucidate the mechanism by which iodide protects against ANTU.

The experiments reported here suggest that differences in certain constituents of the diets of omnivorous and herbivorous animals may play a role in the species variations in susceptibility to ANTU. In addition, however, it is also possible that differences in the metabolism of iodine and in thyroid function may be important in connection with the species variations in susceptibility to ANTU.

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#### SUMMARY

1. Variations in the fat, carbohydrate, and protein, within limits which provided normal or nearly normal growth of rats, did not alter the resistance of rats to ANTU poisoning. Lowering the protein content of the diet to 6% or 0% slightly increased the susceptibility to ANTU.

2. A diet high in cystine provided rats with some protection against ANTU poisoning when the diet was fed for 10 days. If the same diet was fed for longer periods, however, the protective action of cystine was lost until at 30 days no protection remained. It was necessary to provide enough choline in the diet to give normal growth before cystine increased the resistance to ANTU.

3. Potassium iodide or Lugol's solution fed either in the drinking water or in the diet protected rats against large doses of ANTU. Potassium iodide fed at a level of 5.7 mgm. per gram of diet for 10 days provided rats with enough resistance to survive doses of ANTU as high as 100 mgm./kgm.

4. A diet containing 5.7 mgm. of iodine/gram provided thyroidectomized rats with much less protection than it did normal animals against ANTU.

5. The intravenous injection of iodine as diluted Lugol's solution immediately before the administration of ANTU resulted in no observable protection against the rodenticide.

6. A diet containing 12.5 mgm. of desiccated thyroid per gram fed for a 2-day period offered no protection to rats against ANTU.

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## DETOXIFICATION OF BARBITURATES

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The widespread use of barbiturates has attracted considerable interest to the metabolic fate of these compounds. The variation in duration of action of members of this group has usually been attributed to differences in manner of detoxification. It is the purpose of this report to describe observations on the *in vitro* and *in vivo* destruction of certain barbiturates and to correlate these with known observations regarding their pharmacological properties.

A large number of investigators have studied the duration of narcosis produced by various barbiturates on animals previously treated with liver poisons such as phosphorus, chloroform, and carbon tetrachloride. Thus Pratt and coworkers (1, 2) found that rabbits poisoned with phosphorus showed a prolongation of narcosis by pentobarbital but not by barbital. They postulated that pentobarbital is destroyed by the liver but barbital is not. In contrast, Koppányi, Dille, and Linegar (3) found that the action of both sodium barbital and pentobarbital was prolonged in cats and rabbits treated with chloroform. These investigators concluded that this type of experiment was not adequate to determine the site of detoxification, since the direct effect of the toxic agent on the nervous system was not controlled. A number of workers have since confirmed the fact that at least with the "short acting" barbiturates, definite prolongation of narcosis follows treatment with substances known to be toxic to the liver.

Other workers have studied this problem by the use of partial hepatectomy. Thus Schleifley and Higgins (4) found that this procedure caused marked prolongation of anesthesia in rats by ethyl-o-ethylphenylurea and pentobarbital but not by pentothal. They concluded that the former two compounds were broken down by the liver, but the latter is not. Using a similar technique Mason and Beland (5) have studied an extensive series of barbiturates. They obtained evidence indicating that ipral, amytal, pentobarbital, nostal, ortal, alurate, seconal, allyl-pental, evipal, thioethamyl, neonal, delvinal, phanodorn, and dial were detoxified by the liver while barbital, phenobarbital, pentothal, propyl l-methylallyl, and allyl l-methylallyl thiobarbiturates were not broken down by this organ. This is of interest since, in conformity with Schleifley and Higgins (4), the conclusion was reached that pentothal was not degraded by the liver.

A variety of studies have been carried out concerning the role of the kidney in detoxification of barbiturates. The techniques used have not distinguished between destruction and excretion of these compounds. Hirshfelder and Haury (6) found that bilateral nephrectomy in rabbits prolonged the action of phenobarbital and barbital but did not affect the duration of action of amytal, allyl

isopropyl barbituric acid, neonal, ipral, dial, phanodorn, or pernoston. Murphy and Koppányi (7) produced nephrosis in dogs and rabbits by the use of tartaric acid, potassium chromate, and uranium acetate. They found that such treatment resulted in failure to recover from barbital anesthesia but had no effect on anesthesia produced by neonal, sandoptal, pentobarbital, or pernoston. More recently Masson and Beland (5) have reinvestigated this question using bilaterally nephrectomized rats. They concluded that barbital and phenobarbital are detoxified mainly in the kidney while neonal, delvinal, phanodorn, and dial are detoxified approximately equally in the kidney and liver.

Relatively few studies have been concerned with the *in vitro* destruction of barbiturates. Martin, Herrlich, and Clark (8) using macerated tissues, claimed that skeletal muscle, spleen, and liver destroy evipal, while brain, kidney, and blood do not. Determination of barbiturates was based on injection of extracts and could hardly be considered quantitative. Delmonico (9) studied the effect of 24 hour incubation of minced rabbit muscle, kidney, brain, and liver with pentobarbital, sodium amytal, and pentothal. He concluded that all four of these tissues were able to destroy these compounds. The ability of these tissues to degrade all three drugs was in the following order; liver, kidney, skeletal muscle, and brain. These experiments are open to the serious objection in that there is no mention of sterility being maintained, thus making uncertain the role of contaminating microorganisms.

✓ **METHODS.** The harbiturates were determined by an ultra violet spectrophotometric method recently devised in this laboratory (10, 11). This depends on extraction of the drugs from biological materials by redistilled chloroform and determining their absorption of ultra violet light, either in chloroform or 0.5N NaOH, by means of the Beckman Spectrophotometer.

*In vivo* experiments were performed on white mice weighing approximately 20 gm. each. The appropriate drug was injected intraperitoneally and the animals were kept in liter beakers during the experimental period. The animals were killed by a blow on the head and excreta washed out of the beaker with 40 cc of 0.2M acetate buffer (pH = 5.0) and 250 cc of redistilled chloroform. The entire animal plus the buffer and chloroform were then placed in the Waring blender until the animal was completely homogenized. The mixture was centrifuged and the separated chloroform layer was clarified by the addition of 5 gm. of anhydrous sodium sulfate followed by gravity filtration. An aliquot of the chloroform was extracted with 0.5N NaOH and harbiturate was determined spectrophotometrically. Recoveries were performed with all experiments. These were done by killing animals immediately after injection and then using the same procedure as was used with experimental animals. Recoveries were always approximately 95% of injected drug. By analysis of the whole animal together with excreta the total amount of harbiturate degraded could be determined independent of excretion or storage.

Albino rabbits weighing approximately 3 kg. were used for all *in vitro* experiments. The animals were killed by injection of 30 cc of air in the marginal ear vein. Tissues were removed as rapidly as possible and usually less than one hour elapsed between the death of the animals and the beginning of the experiment.

Tissue slices were cut free hand by a razor blade. Breie were prepared by the use of a pair of small scissors. Tissues were homogenized in a glass homogenizer. The tissue slices were weighed while immersed in buffer on a torsion balance.

*In vitro* experiments were performed in 50 cc Erlenmeyer flasks containing a total volume of 5 cc of 0.15M phosphate buffer (pH = 7.0) containing 2% glucose. The flasks were im-

mersed in a water bath at 38° and shaken for three hours. With each experiment known amounts of drug were added after incubation of an aliquot of tissue. Recoveries were at least 95% in all cases. All experiments were repeated twice in duplicate. Although the absolute amount of degradation varied (10-20%) in different experiments the comparative results were constant. All data given are representative of at least four animals.

**EXPERIMENTAL:** The first group of experiments consisted of a study of the ability of the intact mouse to break down certain representative barbiturates. Table 1 illustrates the results obtained in a group of such experiments. It will be noted that both pentothal and seconal are almost entirely destroyed under

TABLE 1  
*In vivo destruction of barbiturates*

All drugs were injected intraperitoneally. Analysis was performed on whole animal plus excreta. Results given are average of two animals. All experiments were done twice.

BARBITURATE	DOSE	TIME AFTER INJECTION	AMOUNT RECOVERED	% DEGRADATION
	<i>mgm. per kilo</i>	<i>hours</i>	<i>mgm. per kilo</i>	
Pentothal. . . . .	100	21	0	100
Seconal . . . . .	150	18	15	90
Phenobarbital . . . . .	150	24	100	33
Barbital . . . . .	150	24	140	10

TABLE 2  
*Relationship of pentothal degradation to method of preparation of tissue*

	WET WT. OF TISSUE	PENTOTHAL ADDED	PENTOTHAL RECOVERED	% DEGRADATION
	<i>mgms</i>	<i>micrograms</i>	<i>micrograms</i>	
Liver slices . . . . .	250	60	27	53
Liver homogenate . . . . .	500	60	56	6
Liver slices . . . . .	250	60	36	40
Liver brei . . . . .	400	60	52	13
Kidney slices . . . . .	250	60	42	30
Kidney brei . . . . .	400	60	47	21

these conditions. A smaller but definitely significant amount of phenobarbital is destroyed. Only slight destruction of barbital occurs. It should be pointed out that in these experiments no attempt to measure excretion separately was made, since excreta were pooled with the animal.

The remaining studies were performed *in vitro* to determine the site of detoxification of various barbiturates. The first of these were conducted in order to determine the manner in which the tissue can best be prepared.

Table 2 illustrates the results of two experiments, the first compares the activity of liver slices and homogenates, and the second compares liver slices to liver brei and kidney slices to kidney brei. It will be noted from these data that homogeni-

zation results in almost complete destruction of the ability of liver to break down pentothal. More activity is retained by brei, but this is not as high as that of slices.

The apparent loss of enzyme activity on homogenization of tissues is not unusual. Many explanations such as dilution of coenzymes and instability of the enzyme on separation from structural components have been advanced to explain this phenomenon. No attempt at further elucidation was made in this study.

The rate of degradation of a given substrate is generally proportional to the concentration of enzyme-substrate complex. This in turn is proportional to concentration of substrate providing the amount of substrate is below that necessary for complete saturation of the enzyme.

Table 3 illustrates two experiments in which the concentration of barbiturate was varied while the amount of enzyme (250 mgm of liver slices) was kept constant. It will be noted that the amount of barbiturate broken down increases

TABLE 3  
*Relationship of barbiturate added to amount of degradation by liver slices*

DRUG ADDED	DRUG RECOVERED	DRUG DEGRADED	% DEGRADED	K
Pentothal				
mM	mM	mM		mM
.023	.002	.021	91	.036
.046	.017	.028	61	.041
.092	.049	.041	49	.027
.184	.127	.057	31	
Seconal				
.023	.012	.011	48	.069
.046	.025	.021	45	.069
.092	.062	.030	32	.054
.184	.140	.044	24	

with concentration approaching a maximum at 0.184 mM. The last column gives the dissociation constant of the enzyme-substrate complex as calculated from these data according to the following equation.

$$K = \frac{(E)(S)}{(ES)}$$

(ES) = Concentration of enzyme-substrate complex

(E) = Concentration of free enzyme

(E) =  $\Sigma E - ES$

(S) = Concentration of substrate

( $\Sigma E$ ) was obtained graphically and is proportional to the rate of reaction at saturation of the enzyme. At 0.184 mM, the error in determining the value of  $\Sigma E$  becomes so important that K cannot be calculated with any validity. (ES) is proportional to the rate of reaction at any given enzyme concentration.

The errors in this type of experiment (use of tissue slices, permeability, extraction of barbiturates, etc.), make an exact determination of  $K$  unlikely, but despite these relative constancy is obtained. The lower value of  $K_{\text{pentothal}}$  than  $K_{\text{seconal}}$  indicates that pentothal is more tightly bound to the enzyme and thus is more rapidly broken down at lower concentrations. These data are of interest as an illustration of the application of this method to a study of the relative rates of detoxification of related drugs.

The next group of experiments were performed to determine the ability of liver slices to break down various representative barbiturates. In order to be sure that the liver slices used on different days were comparable in activity, pentothal or seconal were used in each experiment and compared with each new compound being studied. It is obvious from the data in table 4 that pentothal, seconal,

TABLE 4  
*Degradation of various barbiturates by liver slices*

BARBITURATE	AMOUNT ADDED	AMOUNT RECOVERED	% DEGRADED
	micrograms	micrograms	
Pentothal.....	120	74	38
Seconal.....	120	87	28
Phenobarbital.....	120	120	0
Seconal.....	120	87	28
Amytal.....	120	98	18
Barbital.....	120	120	0
Seconal.....	120	70	41
Pentobarbital.....	120	84	30
Alurate.....	120	106	12
Seconal.....	120	63	47
Nostal.....	120	88	27
Ipral.....	120	120	0

amytal, pentobarbital, and nostal are readily broken down by rabbit liver slices under the conditions employed. Slight destruction of alurate was also obtained. There is no apparent destruction of barbital, phenobarbital, or ipral.

The next studies were concerned with the role of other tissues in the break down of barbiturates. Table 2 shows a comparison of the ability of liver and kidney slices to degrade pentothal. It is apparent that kidney as well as liver destroys pentothal. On a wet weight basis kidney slices has been consistently less active than liver slices.

Table 5 presents the results of a series of experiments comparing the relative abilities of liver and kidney slices to degrade certain representative barbiturates. Pentothal was included in all experiments for reference purposes. It is immediately obvious from these data that only pentothal is degraded by kidney slices

at an appreciable rate. In marked contrast to liver slices, kidney slices do not degrade seconal, amytal, or pentobarbital.

A limited study of other tissues was also carried out. Due to technical difficulties it was not possible to compare brain and muscle to liver according to the slice technique. Accordingly liver brei was compared with muscle and brain brei. Table 6 gives the results of such experiments using three representative barbiturates. There is evidence of slight degradation of pentothal by brain tissue. Although the utilization was small it was consistently obtained. Neither brain

TABLE 5

*Comparison of degradation of various barbiturates by liver and kidney slices*

BARBITURATE	LIVER			KIDNEY		
	Added	Recovered	% Degraded	Added	Recovered	% Degraded
	$\mu g$	$\mu g$	$\mu g$	$\mu g$	$\mu g$	
Pentothal.....	120	60	50	120	90	25.0
Seconal.....	120	70	42	120	118	0.7
Pentothal.....	120	67	45	120	90	25.0
Pentobarbital.....	120	91	24	120	115	4.0
Pentothal.....				120	92	25.0
Amytal.....				120	120	0
Barbital.....				120	120	0
Phenobarbital.....				120	120	0

TABLE 6

*Degradation of barbiturates by liver, brain, and muscle brei*

120 micrograms of appropriate barbiturate added to all vessels

BARBITURATE	LIVER % DESTRUCTION	BRAIN % DESTRUCTION	MUSCLE % DESTRUCTION
Seconal.....	30	0	0
Pentothal.....	53	10	0
Phenobarbital.....	0	0	0

nor muscle brei showed any evidence of degradation of the other barbiturates studied.

DISCUSSION. Previous investigations have indicated that short acting barbiturates such as seconal, nembutal, etc., are detoxified in the liver. For the most part this conclusion has rested on indirect evidence obtained from experiments utilizing liver poisons. The experiments presented in this paper afford direct proof that liver does in fact break down certain barbiturates, apparently attacking the malonyl urea ring. That the ring structure is actually attacked is evidenced by the fact that changes in the side chain alone have little apparent effect on the characteristic absorption maxima of the malonyl urea and malonyl thio-

urea rings which serve as the basis of the analytical method employed (10, 11).

A special case is presented by sodium pentothal which is a thiobarbiturate. Previous workers (4, 5) have concluded that this compound is not detoxified by the liver. This conclusion has been reached on the basis of the failure of partial hepatectomy or liver poisoning to interfere with recovery from pentothal narcosis. The results presented above indicate that the liver can in fact destroy pentothal at a rate comparable to the destruction of other short acting barbiturates. The fate of pentothal differs from the others in that only this compound can be broken down by the kidney and possibly the brain. It is obvious that previous types of experimentation would have missed this point, in that although liver can destroy pentothal, after poisoning or partial hepatectomy, pentothal may still be destroyed by the kidney at an almost comparable rate.

This point has some immediate clinical importance in that there has been some variation in opinion as to the safety of the use of pentothal anesthesia in patients with liver disease. Thus while various investigators have concluded that pentothal is not destroyed by the liver, Mousel and Lundy (12) have reported prolonged anesthesia due to pentothal in a patient with advanced cirrhosis. This might be expected from the results reported in this paper although the degree of prolongation will depend on the extent to which the kidney is able to break down pentothal. A final answer to this question must be obtained from *in vivo* experiments and clinical experience.

Although we have obtained evidence of the destruction of phenobarbital by the intact mouse no evidence of the tissue involved was obtained from our *in vitro* experiments. It is likely that the rate of destruction is too slow to be apparent in these short term experiments.

#### SUMMARY

1. Pentothal and seconal are rapidly destroyed by the intact mouse. Under similar conditions a much smaller degree of degradation of phenobarbital occurs, while there was no evidence of degradation of barbital.

2. Rabbit liver slices degrade pentothal, seconal, amytal, pentobarbital, and to a slight extent alurate. Liver brei shows somewhat less activity while homogenized liver shows no activity.

3. Pentothal is the only barbiturate tested which is degraded by kidney slices.

4. Muscle and brain brei do not destroy any of the barbiturates tested with the exception of slight destruction of pentothal by brain brei.

5. The evidence presented indicates that the kidney breaks down only the malonyl thiourea ring while the liver breaks down both the malonyl thiourea and malonyl urea rings. The speed with which the rings are attacked varies with the nature of the side chains.

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# THE INTRAPERITONEAL TOXICITY OF SOME GLYCOLS, GLYCOL ETHERS, GLYCOL ESTERS, AND PHTHALATES IN MICE\*

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One of the difficulties encountered in the biological assaying of drugs which are insoluble or unstable in water is the selection of a solvent, or a suspension medium, which will not alter the chemical state of the compound under investigation, and which is non-toxic for the test animal under the experimental conditions selected. In regard to the latter, the toxicity of any solvent is a function of the animal used, the route by which the investigated substance is administered, and the quantity of solvent, per kilogram of body weight, to be used as diluent.

Since the albino mouse is one of the most readily available, and, therefore, most frequently used laboratory animals; and since, furthermore, the intraperitoneal route of drug administration is among the simplest and most expedient, particularly in preliminary studies of drugs, it was felt that an investigation of the toxicity of a number of readily obtainable solvents by intraperitoneal injection in mice would be of interest and of value to a large number of investigators.

Although the acute and chronic toxicity of various members of the glycol, glycol ether, glycol ester, and phthalate series have been investigated (1-13), no reports on the effects of the intraperitoneal administration of these substances in mice have been found in the available literature. With this in mind, toxicity studies on Carworth Farms albino mice were conducted with 16 compounds belonging to the previously mentioned series.

**EXPERIMENTAL.** Female, albino, Carworth Farms mice kept on a standard weekly diet of Gaines' dog biscuits, milk bread, meat, peanuts, cabbage or carrots, apples, oranges, and water and weighing between 18 and 27 grams, were injected intraperitoneally with one dose of the pure, undiluted, redistilled solvents under investigation and were observed for 7 days following injection.

On the basis of preliminary screening tests with each solvent, the mice were divided into 6 equal groups, each of which received a different dose, the latter being graduated on a logarithmic scale. Every dose was administered to a minimum of six mice, and each animal was injected according to body weight. In a number of instances the assays were repeated with four mice at each dosage level, the results of the second assay being combined with those of the first for purposes of calculation. Subsequent to the determination of the median lethal dose ( $LD_{50}$ ) by the method of Bliss for small numbers of animals (14), as a final check, a dose corresponding to the  $LD_{50}$  for each compound was administered intraperitoneally to groups of 10 animals.

Gross and microscopic pathological studies were conducted on animals which died during the first 7 days after injection or were sacrificed at the end of the 7 day period of observation.

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Since it was felt that adequate descriptions of the pathological processes following parenteral administration were available in the literature for diethylene glycol, ethylene glycol, ethyl cellosolve, and dioxane (6), these compounds were not intensively studied pathologically. Observations from the literature are, however, included in the tabulation of the data to serve as bases for comparison with those compounds reported in this study. In addition, diethylene glycol, although well covered in the literature (6), was treated as a control compound and was studied thoroughly to provide a further basis for comparison of the pathological data obtained in this study with that in the literature.

Animals for pathological study were necropsied as soon as possible after death, or immediately after being sacrificed with chloroform. Six to 10 mice were examined both grossly and microscopically (at two or more intervals) during the first 7 days after injection of each compound.

Following the gross examinations, organs were fixed in Zenker's solution, embedded in paraffin, sectioned at 5  $\mu$ , and stained with hematoxylin and eosin. Heart, lungs, liver, spleen, and kidneys were examined microscopically in all animals, and pancreas, adrenals, thymus, lymph nodes, bone marrow, sympathetic tissue, gastrointestinal tract, testis, and ovary in a smaller number.

**RESULTS: A. Median Lethal Doses, Minimum, and Maximum Mortality Data.** The median lethal millimolar doses ( $LD_{50}$ 's) in increasing order of the toxicity of the various solvents investigated were as follows: propylene glycol—127.87, diethylene glycol—91.69, ethylene glycol—90.55, triethylene glycol—54.27, dipropylene glycol—33.54, ethyl carbitol—29.14, methyl cellosolve—28.25, ethyl cellosolve—18.97, dimethyl phthalate—18.75, dibutyl phthalate—14.87, ethylene glycol monoacetate—13.93, diethyl phthalate—12.37, dioxane—8.97, glycol diacetate—8.14, butyl carbitol—5.24, and allyl diglycol carbonate—0.98. Further data, including formulas, molecular weights, and specific gravities of the compounds;  $LD_{50}$ 's in ml./kg., gm./kg., and millimoles/kg., with their respective standard errors; and regression line slopes, with their standard errors, are included in table 1. In table 2 there are enumerated for each compound, the highest doses which gave 0 per cent mortality both during the first 24 hours and during the total observation period of 7 days. In addition, this table also contains similar data for the lowest doses which produced 100 per cent mortality.

**B. Pathological Changes.** The pathological changes in any one organ, when present, were essentially the same for all the compounds of this series, although the relative severity of the lesions varied with the solvent and with the survival time of the animal. The observed changes were as follows:

**Heart**—Occasionally, cardiac dilatation was evident during the first 24 hours; there were, however, no gross changes after this time and no microscopic changes at any time.

**Lungs**—Varying degrees of congestion, atelectasis, edema, and hemorrhage were found. The earliest alterations observed were, usually, diffuse congestion and patchy atelectasis, which were succeeded, first, by more extensive atelectasis with patchy edema and, later, by more extensive edema with petechial hemorrhage. Massive pulmonary hemorrhage was rare, and bronchopneumonia was observed in only a few instances. In animals surviving 7 days, the pathological manifestations were decreased in severity but were of essentially the same nature.

TABLE 1

The acute intraperitoneal toxicity of some undiluted glycols, glycol esters, and phthalates in *C. F.*, female mice observed for seven days following injection

NO.	NAME OF COMPOUND (AND NUMBER OF ANIMALS USED IN ASSAY)	FORMULA	MOL. WT.	SPECIFIC GRAVITY	LD <sub>50</sub> ± S.E. ml./kg.	LD <sub>50</sub> ± S.E. g./kg.	LD <sub>50</sub> ± S.E. millimoles/kg.	SLOPE OF REGRESSION LINE ± S.E.	NO.
1	Propylene Glycol (60)	CH <sub>3</sub> OH—CHOH—CH <sub>3</sub>	76.091	1.010	9.36 ± 1.20	0.73 ± 1.25	127.87 ± 16.13	6.38 ± 1.48	1
2	Diethylene Glycol (38)	(HO·CH <sub>2</sub> ·CH <sub>2</sub> ) <sub>2</sub> O	106.120	1.118	8.70 ± 0.30	0.73 ± 0.33	91.69 ± 3.11	20.61 ± 5.91	2
3	Ethylene Glycol (60)	CH <sub>2</sub> OH—CH <sub>2</sub> OH	62.068	1.115	5.01 ± 1.52	5.62 ± 0.17	90.55 ± 2.71	15.42 ± 1.11	3
4	Triethylene Glycol (38)	CH <sub>2</sub> OH—HOCH <sub>2</sub> CH <sub>2</sub> OCH <sub>2</sub> CH <sub>2</sub> OH	150.172	1.125	7.21 ± 0.25	8.15 ± 0.28	51.27 ± 1.86	18.86 ± 5.58	4
5	Dipropylene Glycol (60)	HOCH <sub>2</sub> CH <sub>2</sub> CH <sub>2</sub> O—CH <sub>2</sub> CH <sub>2</sub> CH <sub>2</sub> OH	134.172	1.025	4.39 ± 0.51	4.50 ± 0.52	33.51 ± 3.88	5.00 ± 3.01	5
6	Ethyl Carbitol (40)	C <sub>2</sub> H <sub>5</sub> OCH <sub>2</sub> CH <sub>2</sub> —OCH <sub>2</sub> CH <sub>2</sub> OH	134.172	0.990	3.95 ± 0.21	3.91 ± 0.21	29.14 ± 1.57	8.80 ± 2.65	6
7	Methyl Cellosolve (57)	CH <sub>3</sub> OH—CH <sub>2</sub> O—CH <sub>3</sub>	76.091	0.965	2.23 ± 0.07	2.15 ± 0.068	28.25 ± 0.89	17.01 ± 4.17	7
8	Ethyl Cellosolve (60)	CH <sub>3</sub> OH—CH <sub>2</sub> O—C <sub>2</sub> H <sub>5</sub>	90.120	0.936	1.83 ± 0.11	1.71 ± 0.107	18.97 ± 1.19	7.13 ± 1.36	8
9	Dimethyl Phthalate (37)	C <sub>6</sub> H <sub>4</sub> (COOCH <sub>3</sub> ) <sub>2</sub>	194.189	1.189	3.06 ± 0.18	3.61 ± 0.21	18.75 ± 1.08	11.49 ± 5.91	9
10	Dibutyl Phthalate (60)	C <sub>4</sub> H <sub>9</sub> (COOC <sub>4</sub> H <sub>9</sub> ) <sub>2</sub>	278.336	1.015	3.96 ± 0.41	4.11 ± 0.13	11.87 ± 1.51	5.67 ± 1.98	10
11	Ethylene Glycol Monoacetate (36)	HOCH <sub>2</sub> ·CH <sub>2</sub> O·CO·CH <sub>3</sub>	101.100	1.108	1.31 ± 0.15	1.45 ± 0.17	13.93 ± 1.63	8.13 ± 1.97	11
12	Diethyl Phthalate (60)	C <sub>6</sub> H <sub>4</sub> (COOC <sub>2</sub> H <sub>5</sub> ) <sub>2</sub>	222.232	1.121	2.45 ± 0.10	2.75 ± 0.11	12.37 ± 0.19	17.37 ± 5.33	12
13	Dioxane (60)	O(CH <sub>2</sub> CH <sub>2</sub> ) <sub>4</sub> O	88.100	1.031	0.76 ± 0.11	0.79 ± 0.11	8.97 ± 1.59	1.61 ± 2.50	13
14	Glycol Diacetate (60)	CH <sub>2</sub> CO <sub>2</sub> CH <sub>3</sub>	146.110	1.169	1.07 ± 0.45	1.19 ± 0.05	8.11 ± 0.31	15.40 ± 5.31	14
15	Butyl Carbitol (60)	C <sub>4</sub> H <sub>9</sub> OCH <sub>2</sub> CH <sub>2</sub> —OCH <sub>2</sub> CH <sub>2</sub> OH	162.224	0.956	0.89 ± 0.12	0.85 ± 0.11	5.21 ± 0.68	6.69 ± 2.80	15
16	Allyl Diglycol Carbonate (58)	(CH <sub>2</sub> CH <sub>2</sub> OCOOCCH <sub>2</sub> —CH=CH <sub>2</sub> ) <sub>2</sub> O	274.261	1.113	0.21 ± 0.010	0.27 ± 0.018	0.981 ± 0.066	8.82 ± 2.28	16

Liver—Varying degrees of congestion were commonly seen during the first few days. Those compounds which caused lymphoid tissue damage produced similar injury to the lymphoid follicles around the larger ducts and blood vessels. Similarly, leukocytosis was accompanied by an abnormally high number of white cells in the sinusoids. In a few animals, there was pyknosis of hepatic cells during the early stages, and in a still smaller number, there was evidence of mild necrosis. No compound, however, produced the degree of liver damage reported elsewhere for dioxane and some related compounds (6), although in several cases, there was a marked increase in the number of mitotic figures visible in the liver at the end of 7 days. This effect, which was observed also

TABLE 2

*Volumetric Doses Resulting in Zero and in 100 Per Cent Mortality During the First 24 Hours and During 7 Days Following Intraperitoneal Injection of the Various Solvents in Mice*

COMPOUNDS IN ORDER OF INCREASING MOLAR TOXICITIES	HIGHEST DOSE WHICH GAVE 0 MORTALITY DURING 1ST 24 HOURS	HIGHEST DOSE WHICH GAVE 0 MORTALITY DURING 7 DAY PERIOD	LOWEST DOSE WHICH GAVE 100% MORTALITY DURING 1ST 24 HOURS	LOWEST DOSE WHICH GAVE 100% MORTALITY DURING 7 DAY PERIOD
	ml./kg.	ml./kg.	ml./kg.	ml./kg.
1. Propylene Glycol	7.37	3.69	20.84	14.70
2. Diethylene Glycol	7.38	4.94	—	—
3. Ethylene Glycol	4.49	3.63	6.16	6.16
4. Triethylene Glycol	5.34	—	—	7.98
5. Dipropylene Glycol	4.88	—	—	—
6. Ethyl Carbitol	3.61	2.93	—	—
7. Methyl Cellosolve	1.80	1.80	—	—
8. Ethyl Cellosolve	1.59	—	—	2.24
9. Dimethyl Phthalate	2.50	—	4.34	4.34
10. Dibutyl Phthalate	2.40	2.05	—	4.80
11. Ethylene Glycol Monoacetate	1.60	—	—	1.90
12. Diethyl Phthalate	1.70	1.70	3.40	3.40
13. Dioxane	1.65	—	—	1.65
14. Glycol Diacetate	0.80	0.80	—	—
15. Butyl Carbitol	1.31	—	—	1.03
16. Allyl Diglycol Carbonate	0.19	0.15	0.37	0.37

in the lymphoid tissue and gastrointestinal tract, could not be related to earlier pyknosis or necrosis of hepatic cells.

Spleen—With most of the solvents studied, splenic damage occurred very early after injection and consisted of pyknosis, degeneration, and fragmentation of the lymphocytes of the malpighian follicles, followed by phagocytosis of debris and reticulum cell proliferation. The red pulp showed congestion, lesser amounts of lymphocyte fragmentation, and occasionally an increased amount of hemosiderin. By the end of the seventh day, the acute, degenerative phenomena had decreased in severity, and active lymphocyte regeneration, with many mitotic figures, was found in many of the animals.

Kidney—Kidney damage, both glomerular and tubular, occurred with all the

compounds, although there was considerable variation in the relative degrees of damage to glomeruli and tubules among animals as well as among compounds. Protein leakage through the glomerular tufts was accompanied by varying amounts of eosinophilic protein precipitate in the capsular spaces and proximal tubules, and dilatation of the capsular spaces and proximal convoluted tubules. In some cases, the tubular damage developed as rapidly as the glomerular, but more usually it followed the glomerular changes. It consisted of mild to moderate degeneration of the epithelial cells of the proximal convoluted tubules, loops of Henle, and distal convoluted tubules, with sloughing of the cytoplasm into the lumens, vacuolation of the cytoplasm, and nuclear pyknosis. Although small protein casts, composed apparently of transuded plasma protein in some animals, and of renal epithelial cytoplasmic protein in others, were common, no evidence of crystal formation in the tubules, or of tubular obstruction by these casts was seen. Furthermore, tubular necrosis with cast extrusion, as is seen in hemoglobin kidney damage, did not occur. In several animals, small sub-capsular patches of pyelonephritis were found, but this was felt to be an incidental finding, since it has been noted in many animals from the same colony sacrificed in other projects. In no instance was pyelonephritis, or interstitial infiltration, or fibrosis thought to be related to the administration of the compound. While, in some cases, the tubular changes showed significant resolution at the end of 7 days, occasionally moderate to marked epithelial flattening was observed, and, with several compounds, the glomerular protein leakage continued or even increased during this period.

**Pancreas**—Only one compound, allyl diglycol carbonate, caused pancreatic changes. These consisted of swelling of the nuclei of both the acinar and the islet tissues, producing large, vesicular nuclei microscopically resembling glycogen-filled liver nuclei.

**Adrenals**—There were no observable changes in the medullae and no evidence of necrosis or of mitotic abnormalities; the adrenals did exhibit varying degrees of lipid depletion of the cortex.

**Thymus and Lymph Nodes**—These tissues showed essentially the same changes as did the splenic white pulp, with lymphocyte fragmentation followed by reticulum cell proliferation and phagocytosis of cell debris.

**Bone Marrow**—There were no abnormalities in the animals examined.

**Sympathetic Tissue**—No microscopic changes were observed in the retroperitoneal, sympathetic ganglia, or in the aortic body, which was incidentally sectioned in a few cases.

**Gastrointestinal Tract**—Except for edema of the mucosal stroma, and increased mucous secretion in some animals, no abnormalities of the gastrointestinal tract proper were observed during the first few days. However, the lymphoid follicles and the free cells of the wall showed degenerative changes similar to those previously described for compounds producing lymphoid damage.

**Testis**—In all the spermatogenic cell-layers, there was extensive pyknosis during the early stages, but no atrophy or other late effects of testicular damage were found.

**Ovary**—No ovarian abnormalities were observed.

Peritoneum—Fresh hemorrhage, seen in the peritoneal cavities of rare animals dying shortly after injection, was never felt to be of significant degree; microscopic evidences of perisplenitis (apparently chemical) were seen in a slightly larger number dying or sacrificed from the fifth to the seventh day. No correlation of these findings with the drug or dose given could be made, and no gross evidences of reactive peritoneal congestion or effusion were noted in any of the animals autopsied.

In table 3, for each compound investigated, there are graphically listed the relative severity of the major pathological changes observed from the first to the fourth day, inclusive, and from the fifth through the seventh day; in addition, pertinent pathological comments are included for each compound.

Discussion. That any solvent, regardless of its degree of inherent toxicity, may adversely affect the well-being of the animal to some extent is likely when it is considered that the use of a substance in pure form as a diluting medium means the introduction, into the organism, of a considerably hypertonic solution, the action of which may lower the resistance of the host and, thereby, potentiate the toxic action of the assayed material. Moreover, the production of a solvent-engendered hyperemia may result in a more rapid-than-normal drug absorption which may tend to overwhelm the detoxifying mechanisms, again apparently increasing the toxicity inherent in the drug being investigated.

In several instances, despite the fact that only redistilled compounds were used in this study, the pH of the solvents as determined with the glass electrode indicated marked deviations from neutrality towards the acid side. Check assays with neutralized solvents revealed that these deviations, probably the consequence of a low degree of hydrolysis, were not sufficiently great to alter the mortality data originally obtained.

The median lethal doses determined for all compounds were injected into groups of 10 mice which were then observed for the prescribed 7-day period. When the mortalities fell within the ranges ( $P \geq 0.05$ ) calculated from the parameters of the respective dosage-mortality curves, no further toxicity data were gathered. In a few instances, when a check was not obtained, the assays were repeated. The pathological studies revealed that a small number of individuals in some groups of mice had an apparently naturally occurring leukemia which, although it may have altered the survival times of the animals, caused no significant differences in the pathological changes observed; the leukemic infiltrates, in addition, did not seem to differ, in sensitivity to the lymphotoxic action of this series of compounds, from normal lymphoid tissue.

In general, the pathological changes observed in this study agreed with those reported in the literature, with the lungs, lymphoid tissues, and kidneys being the viscera most affected. Less liver damage was observed than has been reported for dioxane and related compounds (6); whether this is due to differences in the times at which pathological studies were done, to the different route of injection, to dietary or other factors, or to differences in the chemical structures of the compounds, is not certain, but the correlation with the literature obtained with diethylene glycol and, based on smaller numbers of animals, with ethylene glycol and dioxane, suggests that the last factor is the significant

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TABLE 3

Relative severity of major pathological changes observed during seven days following the intraperitoneal injection of one lethal or sublethal dose of some glycols, glycol ethers, glycol esters, or phthalates in mice

COMPOUND	FIRST THROUGH FOURTH DAY					FIFTH THROUGH SEVENTH DAY				REMARKS
	Lungs	Spleen and Lymphoid Tissue	Kidneys	Pathological Changes		Lungs	Spleen and Lymphoid Tissue	Kidneys	Pathological Changes	
1. Starvation Controls				None						
2. Propylene Glycol	-	-	++	Pulmonary congestion, hemorrhage, and edema; marked toxic reaction* in spleen and thymus; minimal renal tubular degeneration.		-	+	-		(6)
3. Diethylene Glycol	+	++	+						Hyperactive mitosis in spleen and GI tract; other organs negative.	
4. Diethylene Glycol	+	+	++	Toxic reaction in spleen and thymus; renal glomerular and tubular damage; high white count; pulmonary congestion, and atelectasis.		-	+	-	Regeneration of spleen and lymphoid tissue; no other lesions.	(6)
5. Ethylene Glycol	+	++	+							(6)
6. Triethylene Glycol	+	+	+	Slight renal tubular degeneration.		-	+	+		
7. Dipropylene Glycol	-	-	+						Hyperactive mitosis in spleen, lymphoid tissue, intestinal mucosa, and liver; renal lesions unchanged.	
8. Ethyl Carbitol	-	+	+	Toxic reaction in spleen; mild renal glomerular and tubular degeneration.		-	-	+	Continued kidney damage; lymphoid tissue normal.	
9. Methyl Cellosolve	-	+	+					++	Lymphoid regeneration; renal tubular damage continued.	
10. Ethyl Cellosolve	-	-	+	Pulmonary congestion and atelectasis; toxic reaction in spleen and lymph nodes; renal tubular necrosis.		-	+	-	Hyperactive mitosis in spleen, lymphoid tissue, intestine, and liver; continued renal tubular degeneration and glomerular protein leakage.	(6)
11. Dimethyl Phthalate	+	+	+							

12. Dibutyl Phthalate	++	++	+	Pulmonary congestion, atelectasis, edema, and petechial hemorrhage; toxic reaction in spleen and lymphoid tissue; minimal renal tubular degeneration.	+	+	+	+	Lymphoid regeneration; glomerular protein leakage and continued tubular damage; slight patchy pulmonary edema.
13. Ethylene Glycol Monoacetate	++	+	-	Pulmonary congestion and atelectasis; congestion of viscera.	-	+	+	+	Hyporactive mitosis in lymphoid tissue, liver, and gastrointestinal tract; slight renal tubular degeneration, with small foci of pyelonephritis.
14. Diethyl Phthalate	++	++	++	Pulmonary congestion, edema, and petechial hemorrhage; toxic reaction in spleen; renal tubular degeneration.	+	+	-	+	Continued renal tubular degeneration with flattening of the epithelium.
15. Dioxane	-	-	++	Pulmonary congestion; moderate renal tubular degeneration.	+	+	-	+	Continued renal tubular damage.
16. Glycol Diacetate	++	++	+	Pulmonary congestion and atelectasis; toxic reaction in spleen and lymphoid tissue; glomerular and tubular degeneration; edema of gastrointestinal mucosa plus pulmonary petechial hemorrhage.	+	+	+	+	
17. Butyl Carbitol	+	++	+	Pulmonary congestion, atelectasis, and edema; toxic reaction in spleen and lymphoid tissue; congestion of viscera; renal tubular damage; swelling of pancreatic nuclei.	+	+	+	+	Pulmonary congestion, atelectasis, and patchy bronchopneumonia; glomerular proteinuria; healing hepatic necrosis.
18. Allyl Diglycol Carbonate	++	+	++		+	+	+	+	

- No significant lesions, + Mild Lesions, ++ Moderate Lesions, +++ Severe Lesions.

\* Toxic reaction—cf. pathology of spleen in Results, part B.

onc. Similarly, hemorrhagic nephritis, and oxalate crystalluria, which have been reported to follow administration of ethylene glycol and related compounds (15), were not observed in this study.

#### SUMMARY

The acute, intraperitoneal median lethal doses for 16 solvents belonging to the glycol, glycol ether, glycol ester, and phthalate series were determined in Carworth Farms, female, albino mice, which were observed for 7 days following injection. The  $LD_{50}$ 's, in millimoles per kg., and the pathological changes observed with some of the solvents up to 72 hours were as follows: *propylene glycol* (127.87); *diethylene glycol* (91.69); *ethylene glycol* (90.55); *triethylene glycol* (54.27)—toxic reaction in spleen and thymus, renal glomerular and tubular damage, high white count, pulmonary congestion, and atelectasis; *dipropylene glycol* (33.54)—renal tubular degeneration; *ethyl carbitol* (29.14)—toxic reaction in spleen, renal glomerular and tubular degeneration; *methyl cellosolve* (28.25)—toxic reaction in spleen and lymph nodes, renal tubular degeneration; *ethyl cellosolve* (18.97); *dimethyl phthalate* (18.75)—pulmonary congestion and atelectasis, toxic reaction in spleen and lymph nodes, renal tubular necrosis; *dibutyl phthalate* (14.87)—pulmonary congestion, edema, and petechial hemorrhage, toxic reaction in spleen; renal tubular degeneration; *ethylene glycol monoacetate* (13.93)—pulmonary congestion and atelectasis; *diethyl phthalate* (12.37)—pulmonary congestion, edema, and petechial hemorrhage, toxic reaction in spleen, and renal tubular degeneration; *dioxane* (8.97); *glycol diacetate* (8.14)—pulmonary congestion, renal tubular degeneration; *butyl carbitol* (5.24)—pulmonary congestion and atelectasis, toxic reaction in spleen and lymphoid tissue, glomerular and tubular degeneration; *allyl diglycol carbonate* (0.98)—pulmonary congestion, atelectasis, and edema, toxic reaction in spleen and lymphoid tissue, congestion of viscera, marked renal tubular damage. All fasting controls were negative.

Additional data include formulas, molecular weights, and specific gravities of the compounds;  $LD_{50}$ 's, with their respective standard errors, in ml./kg., gm./kg., and millimoles/kg.; regression line slopes with their standard errors; highest and lowest volumetric doses resulting in 0 and in 100 per cent mortality, respectively, during the first 24 hours following intraperitoneal injection and also for the 7 day observation period; and a tabulation, with pertinent pathological comments, of the relative severity of the major pathological changes observed.

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## L-THIOSORBITOL AND BAL AS ANTIDOTES IN PHENYLTHIOUREA POISONING

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Following the report of the rodenticidal action of ANTU (alpha-naphthylthiourea) and phenylthiourea by Richter (1) much interest has been aroused in these substances. Because of the wide use of these agents, accidental poisoning was anticipated and a search for a suitable antidote was instigated.

Du Bois (2) reported that sulfhydryl-containing compounds were inhibited by ANTU and suggested that an excess of available sulfhydryl radical might protect the essential sulfhydryl-containing compounds of the body.

Peters et al. (3) in England demonstrated the effectiveness of BAL (2,3-dithiolpropanol) as an antidote in arsenic poisoning. Later Gilman et al. (5) found that l-thiosorbitol was also effective in treating poisoning by sulfhydryl-binding substances. In view of this information it seemed logical to study the antidotal effectiveness of BAL and l-thiosorbitol in phenylthiourea poisoning. Since the mode of action of ANTU and phenylthiourea is apparently the same, the latter drug was used in these experiments because of its greater relative solubility.

**EXPERIMENTAL.** Male rats (Wistar strain) weighing approximately 200 grams were used as the test animals. A solution of phenylthiourea (0.60%) in propylene glycol was prepared. Toxicity determinations with propylene glycol showed that as much as 7.5 ml. per Kg. of this solvent alone given intravenously was well tolerated. The average amount of this solvent injected intravenously or intraperitoneally in these experiments was 1.5 ml. per Kg. Eight milligrams of phenylthiourea per Kg. was the intravenous dose used. This dose proved to be an LD<sub>50</sub> since 19 of 28 control rats died. Ten mgs. per Kg. given intraperitoneally was found to be an LD<sub>50</sub>. L-thiosorbitol, which is very soluble in water, was always given intravenously in the form of a 30% or 40% aqueous solution. BAL was injected either intramuscularly or intravenously in the form of a 1.0% solution in propylene glycol. An intravenous injection of 25 mgs. of BAL per Kg. was survived by all of the 8 control rats. Previous work by Eagle (4) and others (6) has shown that doses larger than this are toxic. Fifty (50) mgs. of BAL per Kg. given in a single intramuscular dose killed 2 of 11 rats. All the intravenous injections were made into the tail veins.

**RESULTS.** In table 1 are presented the results of the therapeutic effect of thiosorbitol on poisoning produced by intravenously administered phenylthiourea. One gram of thiosorbitol per Kg. given intravenously immediately after the phenylthiourea increased the percentage of survival from 29.5 in the controls to 92.0 in the treated group. One and one-half grams of thiosorbitol per Kg. given intravenously immediately after the phenylthiourea raised the percentage of survival from 29.5 to 83.0. When one hour was allowed to elapse between the administration of the phenylthiourea and the intravenous dose of one gram of thiosorbitol per Kg. there was no evident antidotal effect.

Table 1 also presents data which indicate that intravenous thiosorbitol failed to protect rats poisoned intraperitoneally with an  $LD_{50}$  of phenylthiourea when a one-half hour interval elapsed between the poisoning and the treatment.

In table 2 are presented the results of the therapeutic effect of BAL on poisoning produced by intravenously administered phenylthiourea. Twenty-five (25) mgs. of BAL per Kg. given intravenously immediately after phenylthiourea resulted in a higher mortality rate than did the phenylthiourea alone. The same result was obtained when the phenylthiourea was followed immediately by 25 mgs. of BAL per Kg. intramuscularly and by a second and similar amount

TABLE 1

*Treatment of phenylthiourea poisoning with intravenous thiosorbitol*

TREATMENT (THIOSORBITOL)	NO. OF RATS	NO. OF SURVIVORS	PERCENTAGE OF SURVIVAL
(Intravenous phenylthiourea— $LD_{70}$ )			
1.0 gm./Kg. immediately ..	50	46	92
1.5 gms./Kg. immediately	12	10	83
1.0 gm./Kg. one hour after poisoning .	23	7	30
(Intraperitoneal phenylthiourea— $LD_{50}$ )			
1.0 gm./Kg. one-half hour after poisoning	16	0	0

TABLE 2

*Treatment of intravenous phenylthiourea poisoning ( $LD_{70}$ ) with BAL*

TREATMENT (BAL)	NO OF RATS	NO. OF SURVIVORS	PERCENTAGE OF SURVIVAL
25 mgs./Kg. immediately intravenously .	6	1	16
50 mgs./Kg. (25 mgs. immediately) (25 mgs. 1 hour later) intramuscularly	20	3	15
75 mgs./Kg. (25 mgs. immediately) (50 mgs. 1 hour later) intramuscularly.	12	3	25

of BAL one hour later. In the third group presented in table 2 the rats received 25 mgs. of BAL per Kg. intramuscularly immediately after the phenylthiourea was administered and an additional 50 mgs. of BAL per Kg. by the same route one hour later. In this group also, the mortality rate was higher than that of the untreated controls.

DISCUSSION. The results of the experiments presented here indicate that protection is afforded by l-thiosorbitol when given immediately following poisoning by phenylthiourea, but that if therapy is delayed for 30 minutes or longer no apparent antidotal action is evident. These findings are in agreement with those of both the British and American workers (3, 4, 5) who have investigated

the ability of thiols to reverse the effects of arsenic and cadmium poisoning. Both groups found the monothiols to be ineffective unless given immediately after the poisoning.

BAL produced an increase rather than a decrease in the mortality rate of phenylthiourea poisoning. This occurred when BAL was given either as an immediate single dose or in divided doses, one immediately and another one hour after the phenylthiourea was administered.

This successful protection with thiosorbitol and the failure of BAL to protect are the same results obtained when cadmium poisoning is treated with single doses of these two thiols (5). It is possible that the reason for this discrepancy would be disclosed by investigations along the lines suggested by Gilman et al. (5) concerning the mode of action of BAL in cadmium poisoning. They postulated that since renal tubular damage was produced by the Cd-BAL complex and not by the Cd-thiosorbitol complex, the former was absorbed by the tubular epithelium and the BAL was oxidized sufficiently by the epithelial cells to free the toxic  $Cd^{++}$  which then damaged the tubular epithelium. The Cd-thiosorbitol complex, on the other hand, was not absorbed by the tubule but was excreted by the kidney in a similar manner as certain polyhydric alcohols such as sorbitol and mannitol. These polyhydric alcohols are filtered through the glomerulus and are not reabsorbed by the tubules. Reasoning by analogy, it is possible that if BAL and phenylthiourea do temporarily unite in vivo, the complex is filtered through the glomerulus and is reabsorbed by the tubule. The BAL could then be oxidized and the phenylthiourea thus liberated continue its toxic effects on the lungs. To extend the analogy, the possibility also exists that the theoretical thiosorbitol-thiourea complex is non-toxic because it remains extracellular in all tissues, while the BAL-thiourea unit can easily enter some cells such as those of the pulmonary epithelium, the BAL then be oxidized, and the thiourea freed to exert its poisonous effects. On the other hand BAL alone has been shown by Modell et al. (6) to produce pulmonary edema.

#### SUMMARY AND CONCLUSIONS

1. Thiosorbitol is an effective antidote for intravenously administered phenylthiourea if it is given by the same route and immediately following the phenylthiourea.

2. No antidotal effect is observed when thiosorbitol is given 30 minutes or longer after the administration of phenylthiourea.

3. BAL increases rather than decreases the toxic effect of phenylthiourea.

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# THE PHARMACOLOGY OF 2-AMINO-6-METHYLHEPTANE<sup>1</sup>

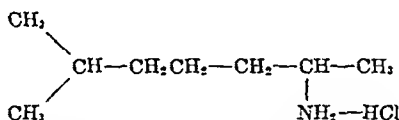
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In 1910 Barger and Dale (1) reported that a number of phenylethyl- and aliphatic-amines exhibited physiological effects which they designated as sympathomimetic. This type of action subsequently was found to be inherent in a number of phenylisopropylamine derivatives (2, 3). While most of the activity in this field has been concerned with studies on aralkylamines, considerable attention has been devoted to aliphatic amines (4, 5, 6, 7, 8, 9, 10, 11, 12) in recent years. Most of the sympathomimetic amines have been appraised primarily for vasoconstrictor, bronchodilator or analeptic activity, however, certain of these agents recently have been reported to exhibit such totally unexpected actions as analgetic and local anesthetic (13, 14, 15, 16).

In the course of a systematic study of alkyl- and aralkyl-amines, the following aliphatic derivative was found to exhibit a number of interesting physiological effects:



2-Amino-6-Methylheptane Hydrochloride (Code S-51)

**ANALGESIA.** The effect of 2-amino-6-methylheptane (S-51)<sup>2</sup> hydrochloride on threshold pain stimulus in cats was determined by a method similar to that described by Eddy (17). The variations in the normal thresholds by this method were found to be of the order of 25 per cent. In the present studies, significant activity was inferred if thresholds were elevated 100 per cent or more by a given dose. This amount was not considered definitely analgetic unless thresholds were elevated in at least 4 out of 5 cats. As shown in table 1, doses of 3.0, 5.0, 7.5 and 10.0 mgm. per kilogram of body weight<sup>3</sup> induced a significant and consistent elevation of threshold pain stimulus without producing excessive side effects. While a marked elevation of threshold pain stimulus was observed after 15.0 mgm., side effects were pronounced.

**LOCAL ANESTHESIA.** The topical local anesthetic activity of S-51 was determined in rabbits using the method previously described (18). As shown in

<sup>1</sup>This investigation was supported in a large part by the Smith, Kline and French Laboratories Fellowship Fund.

<sup>2</sup>In order to facilitate discussion throughout the remainder of the text, it is understood that S-51 refers to the hydrochloride salt of 2-amino-6-methylheptane unless otherwise specified.

<sup>3</sup>Throughout the remainder of the text it is understood that all doses mentioned are per kilogram of body weight unless otherwise noted.



table 2, 1.0 per cent solution of S-51 produced anesthesia for an average of 13.1 minutes. A stream of air was passed over a pledget containing the free base of S-51, which is volatile, and subsequently allowed to come in contact with a rabbit's eye. This procedure was found to induce local anesthesia, but did not permit quantitative evaluation. Therefore, the local anesthetic activity of

TABLE 1

*Analgesia*

Effect of 2-amino-6-methylheptane hydrochloride (S-51) on threshold pain stimulus, after intraperitoneal injection in cats.

DOSE	NUMBER/NUMBER ANALG /USED	MAXIMUM INCREASE IN PAIN THRESHOLD	COMMENTS
<i>mgm /kgm.</i>		<i>per cent</i>	
15.0	5/5	378, 388, 194, 294, 301	Convulsions-1/5 General. tremors-3/5
10.0	10/10	83, 182, 63, 140, 281, 246 63, 218, 93, 225	Convulsions-1/10 Salivated-7/10 Panted-5/10
7.5	4/5	160, 120, 116, 218	No side effects-2/5 Hyperpnea-3/5
5.0	4/5	141, 175, 213, 183	Panted-1/5 No side effects-4/5
3.0	5/5	131, 121, 145, 119, 148	Dilated pupils-5/5 Vomited-1/5 Hyperpnea-2/5
2.0	2/5	35, 50	Dilated pupils-5/5

TABLE 2

*Local anesthesia*

Topical application of 2-amino-6-methylheptane hydrochloride (S-51), free base, or cocaine hydrochloride to rabbits' eyes.

COMPOUND	NUMBER OF TESTS	CONCENTRATION	AVERAGE DURATION OF ANESTHESIA
		<i>per cent</i>	<i>minutes</i>
S-51 HCl	26	1.0	13.1
S-51 (free base)	21	0.2	14.1
S-51 (free base)	19	0.1	7.1
Cocaine HCl	20	1.0	19.5

S-51 (free base) was appraised in aqueous solution and as shown in table 2, in 0.1 and 0.2 per cent concentration produced anesthesia for an average of 7.1 and 14.1 minutes respectively. Under present conditions, 1.0 per cent solution of cocaine hydrochloride exhibited local anesthetic activity for an average of 19.5 minutes.

The fact that S-51 was found to diminish the excitability of certain nerve structures is interesting because the volatile free base of this substance might be utilized to decrease irritability of certain accessible afferent nerves such as those in the tracheo-bronchial tree. It appeared desirable, therefore, to determine whether or not the possible usefulness of S-51 would be complicated by marked systemic effects or toxic properties.

**CIRCULATORY ACTION.** The circulatory activity of S-51 was appraised in 14 vagotomized atropinized dogs anesthetized with pentobarbital sodium, and standardized with epinephrine. In these experiments, the pressor activity of 0.000006–0.000003 millimol of epinephrine was approximated by 0.003 millimol of S-51, therefore, its intravenous pressor activity is 1/500–1/1000 that of epinephrine. Since tachyphylaxis is readily apparent after S-51, only one dose of this substance was injected in a given animal in all of the experiments involving determination of epinephrine equivalents.

The effect of intravenous injection of 0.5 to 1.0 mgm. of S-51 was studied in 8 vagotomized dogs anesthetized with pentobarbital sodium in which the spleen, a kidney, a section of small intestine and a leg were enclosed in plethysmographs. In these experiments, a negligible decrease in spleen, an increase in kidney, leg and intestinal volume were noted. In 4 experiments on vagotomized dogs anesthetized with pentobarbital sodium, 1.0 mgm. of S-51 caused an increase in rate and amplitude of contraction of the heart as determined by a Cushney Myocardiograph.

**MISCELLANEOUS EFFECTS IN ANESTHETIZED DOGS.** In 15 experiments on dogs anesthetized with pentobarbital sodium, intravenous injection of 0.5 to 1.0 mgm. of S-51 failed to produce a detectable effect on the small intestine, detrusor of the urinary bladder, urine secretion, respiration, vagus sensitivity, circulatory response to acetylcholine, furfuryltrimethyl ammonium iodide or epinephrine.

**ISOLATED INTESTINE.** The effect of concentrations of 1:10,000 to 1:200,000 of S-51 was determined in 9 experiments on isolated segments of rabbit jejunum. In general, relaxation occurred throughout the entire dose range used. A slight and transient effect was noted in 1:200,000, whereas marked and prolonged relaxation was observed in 1:10,000 concentration. In two experiments slight augmentation of the intestine was noted during exposure to 1:50,000 S-51.

**MOTOR ACTIVITY.** A method of detecting and summing spontaneous activity of rats has been developed and utilized to measure the effect of a number of sympathomimetic amines on the central nervous system (19, 20). Stimulation was inferred if motor activity increased after administration of a given compound. A record of the activity of a normal rat is illustrated in Fig. 1, A. Fig. 1, B is a kymographic tracing which illustrates the effect of 1.5 mgm. intraperitoneally of amphetamine sulfate on motor activity in rats. The increase in the number of vertical lines in this case indicates for the most part frequent movement about the cage. Doses of 3.0 mgm. of amphetamine sulfate intraperitoneally produced a typical effect (Fig. 1, C). This consisted almost entirely of side-to-side head motion with little, if any, movement about the cage. S-51 was administered in doses of 20, 25 and 35 mgm. (Fig. 1, D, E, and F) intraperitoneally and 150 mgm. (Fig. 1, G) orally. While there is an indication of more activity after

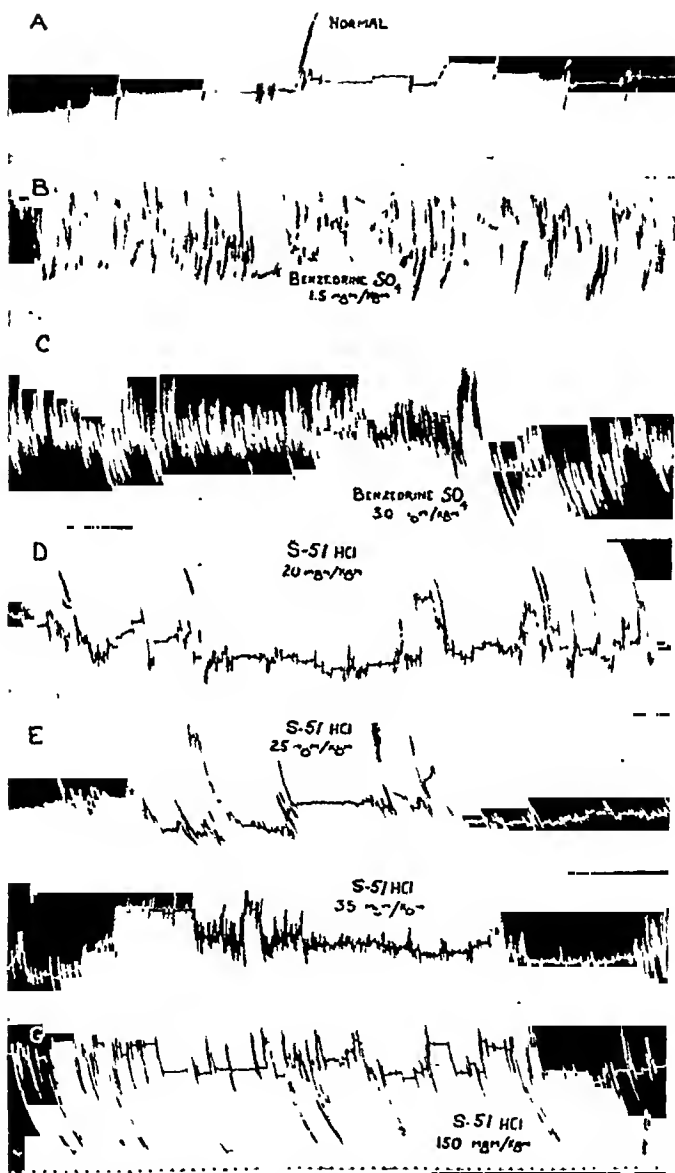


FIG. 1. Motor activity of Rats. A Normal activity, B Activity after 1.5 mgm. Amphetamine Sulfate intraperitoneally; C Activity after 30 mgm. Amphetamine Sulfate intraperitoneally; D, E, F. Activity after 20, 25 and 35 mgm. of 2-amino-6-methylheptane hydrochloride (S-51) intraperitoneally; G. Activity after 150 mgm. of S-51 orally. All doses are mgm. per kilogram of body weight.

S-51 than that observed in normal rats, a typical "amphetamine-like" effect was never noted.

**ACUTE TOXICITY.** *Mice:* Doses of 40 to 75 mgm. of S-51 were injected intraperitoneally in mice (table 3). From the observed mortality shown in table 3, the LD<sub>50</sub> was estimated to be 59 mgm.

TABLE 3

*Acute toxicity*

Intraperitoneal injection of 2-amino-6-methylheptane hydrochloride (S-51)

ANIMAL	DOSE mgm./kgm.	MORTALITY RATIO	OBSERVATIONS
Mice	40	0/20	Restlessness-20/20
	50	1/25	Depression followed by excitement-25/25
	65	13/20	Tremors and convulsions-20/20
	75	19/20	Convulsions-20/20
			Death 10 minutes after injection-19/20
Rats	30	0/10	Tremors-4/10
			Increased activity-10/10
	40	4/10	Convulsions-8/10
	50	10/10	Tremors, salivation, increased activity-10/10
Rabbits	60	10/10	Convulsions and death within 20 minutes
			Convulsions and death within 10 minutes.
	50	0/6	Tremors-2/6
	60	3/6	Convulsions and tremors-3/6
	75	4/6	Convulsions-4/6
Guinea pigs	100	6/6	Tremors-1/6
			Convulsions and death within 7-8 minutes.
	25	0/6	Slight tremors-6/6
	35	2/6	Convulsions-2/6
	50	6/6	Excitement-6/6
	75	6/6	Convulsions and death 1 hour after injection
	100	6/6	Convulsions and death ½ hour after injection
			Tremors, convulsions-6/6
			Death 12-15 minutes after injection.

*Rats:* S-51 was injected intraperitoneally in doses of 30 to 60 mgm., the mortality ratio shown in table 3 obtained and the estimated LD<sub>50</sub> found to be 41.5 mgm.

*Rabbits:* Intraperitoneal injections of 40 to 100 mgm. of S-51 were made in rabbits (table 3). From the mortality data shown in table 3, an estimated LD<sub>50</sub> of 44 mgm. was obtained.

*Guinea Pigs:* In these animals 25 to 100 mgm. of S-51 were injected intraperitoneally (table 3). From these data, an LD<sub>50</sub> of 39 mgm. was estimated.

**SUBACUTE TOXICITY.** Each of 10 rats (Table 4) received daily intraperitoneal injections of 20 mgm. (approximately one-half the LD<sub>50</sub>) of S-51 for 30 days.

At the end of this period these animals showed an average weight gain of 38 grams.

Nine rats (Table 4) were given daily oral doses of 100 mgm. of S-51 for 30 days. After each administration all rats evidenced depression which was followed by piloerection and restlessness. These animals showed an average weight gain of 38 grams. Six other rats (Table 4) received oral doses of 75 mgm. of S-51 for 30 days. The only effect noted in these animals was slight depression. The average weight gain for this group of animals was 49 grams.

Twenty-five mgm. (slightly in excess of one-half the  $LD_{50}$ ) of S-51 were injected intraperitoneally in each of six guinea-pigs (Table 4) daily for a 30-day interval. All of the animals in this group evidenced slight tremors during the first few days of the injection period, but after several days had elapsed, slight restlessness was the only symptom noted. Six other guinea-pigs received daily intraperitoneal injections of 20 mgm. (One-half the  $LD_{50}$ ) of S-51 for 30 days.

TABLE 4  
*Subacute toxicity*

Oral and intraperitoneal administration of 2-amino-6-methylheptane hydrochloride (S-51) daily for 30 days.

ANIMAL	NUMBER OF ANIMALS	ROUTE OF ADMINISTRATION	DOSE	AVERAGE WEIGHT BEFORE INJECTION	AVERAGE WEIGHT END OF 30 DAYS
			mgm./kgm.	kgm.	kgm.
Rats.....	9	P.O.	100.0	0.118	0.156
Rats.....	6	P.O.	75.0	0.109	0.158
Rats.....	10	Ip.	20.0	0.143	0.181
Guinea pigs.....	6	Ip.	20.0	0.254	0.336
Guinea pigs.....	6	Ip.	25.0	0.242	0.322
Rabbits.....	8	Ip.	20.0	2.07	2.14

All guinea-pigs receiving either the 20 or 25 mgm. dose gained weight throughout the period of administration (Table 4).

Each of 8 rabbits received daily doses of 20 mgm. (approximately one half the  $LD_{50}$ ) of S-51 for 30 days. These animals evidenced little change in weight during the 30-day administration period (Table 4).

At the end of the 30 day period of administration all of the animals in Table 4 were sacrificed and sections of heart, lung, liver, kidney, spleen, intestine stomach and the entire brain and cord were removed. Examination of these specimens by Dr. Charles F. Branch revealed that S-51 did not produce tissue damage which could be detected histologically.

A number of experiments were carried out in which S-51 (free base) was inhaled by rabbits and rats. The primary purpose of these studies was to determine whether or not this material would produce irritation of the respiratory tract mucosa. Twenty rabbits were immobilized and arranged to inhale fixed doses of 5.0 to 10.0 mgm. of S-51 (free base) daily for a 10-day period. Twelve rats were placed in a gas chamber and subjected to continuous inhalation of

S-51 (free base) plus flavoring agents for 6 hours daily for 30 days. The air flow through the S-51 (free base) mixture was adjusted to a rate of 6 liters per minute in such a manner that 0.4 to 0.55 mgm. of S-51 (free base) were delivered in each liter of air throughout the entire six hour period. At the expiration of the 10 and 30 day period respectively, the rabbits and rats were sacrificed and the entire trachea, lungs, brain and cord as well as sections of kidney, spleen, intestine and stomach were removed. Examination of these tissues by Dr. Charles F. Branch disclosed that S-51 did not induce histopathologically demonstrable damage.

#### SUMMARY

1. The threshold pain stimulus was elevated in cats after doses of 3.0-15.0 mgm. per kilogram of body weight of 2-amino-6-methylheptane hydrochloride intraperitoneally.

2. Local anesthesia was produced by topical application of solutions of the hydrochloride or free base of 2-amino-6-methylheptane to rabbit's eyes.

3. Intravenously in dogs anesthetized with pentobarbital sodium, 2-amino-6-methylheptane hydrochloride exhibited 1/500-1/1000 the pressor activity of epinephrine.

4. As determined with a Cushny Myocardiograph, 2-amino-6-methylheptane hydrochloride was found to cause an increase in cardiac rate and amplitude of contraction.

5. Intravenously in dogs, anesthetized with pentobarbital sodium, doses of 0.5 to 1.0 mgm. per kilogram of body weight of 2-amino-6-methylheptane hydrochloride had no detectable effect on the small intestine, detrusor of the urinary bladder, urine secretion or respiration.

6. In general, concentrations of 1:10,000 to 1:200,000 of 2-amino-6-methylheptane hydrochloride caused relaxation of isolated segments of rabbit jejunum.

7. Intraperitoneally in rats, 2-amino-6-methylheptane hydrochloride was found to be almost entirely devoid of a central nervous system stimulating action in non-toxic doses.

8. Acute toxicity data were obtained on 2-amino-6-methylheptane hydrochloride in mice, rats, rabbits and guinea-pigs.

9. Large doses of 2-amino-6-methylheptane hydrochloride were administered daily for 30 days to rats, rabbits, and guinea-pigs. Examination of the tissues removed from these animals disclosed that this compound did not induce histopathologically demonstrable damage.

10. No histological evidence of irritation of the tracheo-bronchial tree mucosa was noted in rabbits or rats which inhaled 2-amino-6-methylheptane free base daily for 10 and 30 days respectively.

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# EFFECT OF RUTIN ON PERMEABILITY OF CUTANEOUS CAPILLARIES\*

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At the present writing quite an extensive literature exists on the use of rutin, flavonols and flavone derivatives in clinical conditions characterized by increased permeability and fragility of the capillaries. One of the difficulties encountered in evaluating the "vitamin P" potency of flavonols and flavone derivatives both clinically and in experimental animals has been the lack of pure materials. Fortunately the isolation of rutin from buckwheat by the Eastern Regional Research Laboratory (1) has provided a readily available source of a pure crystalline compound which can be investigated exhaustively under clinical and experimental conditions.

In 1936, the use of "citrin" or vitamin P was reported by Armentano, Bentsáth, Béres, Rusznyák, and Szent-Györgyi (2) to have some beneficial effect in man by increasing the resistance of the capillary wall. Similar observations were made by Scarborough (3), using "citrin" and hesperidin, and Goldfarb (4) used "citrin" and ascorbic acid in the treatment of psoriasis. More recently, Griffith, Couch, and Lindauer (5) and Shanno (6) have reported that rutin, a rhamnoglucoside of the flavonol quercetin, increases the resistance of the capillary wall.

A clear differentiation between fragility and permeability changes of the capillaries has not been made and some confusion in the use of the terms exists in the literature. The demonstration of a protective action by vitamin P against capillary damage in scorbutic guinea pigs has been attempted by a number of investigators, and Bacharach, Coates and Middleton (7) have proposed a bioassay method for vitamin P. Essentially, the method is based upon a comparison of the negative pressure values at which petechiae first appear in control and treated guinea pigs. This criterion apparently involves an actual break of the capillaries. Likewise the determination of the petechial index in clinical cases by Griffith and co-workers (5), and by Shanno (6) is probably a measure of the breaking of capillaries.

Griffith, Lindauer, Shanno and Couch (8) have studied lymph flow in patients by observing the spread of the blue colloidal dye; patent blue, following its injection into the antecubital space, an increasing spread of the dye being considered as indicative of an increased lymph flow. These authors point out that increased cutaneous lymph flow indicates passage of fluid through the capillary wall, due to either increased capillary pressure or increased capillary permeability,

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and it is probable that increased permeability is often associated with increased fragility.

Attempts in this laboratory (9) to demonstrate a protective action on the capillaries of scorbutic guinea pigs by means of rutin have failed. Attention has, therefore, been turned to the development of a method of evaluation based upon permeability changes rather than increased fragility.

**METHOD.** It has been observed previously by others that the application to the skin of an irritant such as chloroform, or the intracutaneous injection of histamine, results in the accumulation of intravenously injected trypan blue in the areas of inflammation. The local staining at the site of inflammation has been used quite extensively as a means of demonstrating local changes in capillary permeability. For a discussion and review of the literature see Menkin (10).

The permeability of the cutaneous capillaries was observed in 22 albino rabbits weighing 2 to 4 kilograms each and maintained on Purina Rabbit Chow.<sup>1</sup> The ventral surface, from the symphysis pubis to the xiphoid process and extending laterally for about 4 cm. on each side of the mid-line, was depilated with barium sulfide 24 hours before the observations were made. On the day of the test the rabbits were fastened back downward on animal boards and the depilated surface marked off into 10 approximately equal squares. Each rabbit was given intravenously, by way of the marginal ear vein, 2 ml. of a 1 percent trypan blue solution.

Approximately 5 minutes later chloroform was applied to the skin by means of a cotton-tipped applicator (total diameter about 5 mm.). The applicator was dipped in chloroform, the excess removed by shaking, and the moistened applicator applied to the skin with slight pressure for 30 seconds. Usually these applications were made in pairs on each rabbit, the applications being on each side of the mid-line and toward opposite ends of the depilated surface. The lapse of time between application of the chloroform and the first definite appearance of dye in the irritated area was noted. As soon as this time was determined, 0.25, 0.5, or 1 ml. per kilogram of body weight of a 20 percent solution of rutin in propylene glycol diluted with an equal volume of 0.9 percent sodium chloride or Ringer-Locke solution was injected intravenously by way of the marginal ear vein.

A 20 percent solution of rutin in propylene glycol was too viscous for intravenous injection and therefore was diluted with an equal volume of saline. Such a diluted solution will become cloudy in a few minutes and somewhat later a precipitate of rutin settles out. However, when the solution was injected slowly immediately after being prepared none of the rabbits showed anaphylactoid symptoms.

After injection of the rutin, chloroform was applied to new areas at approximately 5-minute intervals, and in some cases repeated applications were made until the time of dye appearance in the irritated area returned to the control values. Where observations were made for more than one hour, additional injections of 1 ml. of trypan blue solution were made every hour.

For the purpose of comparing the results produced by chloroform irritation with other procedures for producing capillary damage, histamine wheals were produced in 3 of the rabbits, and in 6 others 30 mm. of negative pressure was applied to the depilated area for one minute by means of a standardized cup before and after the administration of rutin. Only two of the three procedures were applied to any one animal at any one time. Each individual procedure was carried out in duplicate and repeated to ensure that the observed responses were characteristic and reproducible.

**RESULTS.** In table 1 data are presented on the dye-appearance time in chloroform-irritated areas, in histamine wheals and in areas under negative pressure.

<sup>1</sup> Purina Rabbit Chow is specified as a part of the experimental conditions and not because it is regarded as superior or inferior to other commercial diets.

Before the administration of rutin the accumulation of the dye in the chloroform-irritated areas of 19 rabbits appeared in from 1 to 6 minutes. After rutin, the appearance of the dye in newly irritated areas occurred as early as 4 minutes and was delayed as much as 54 minutes. It is important to note that while there is considerable variation in the response of different rabbits each animal served as its own control, and that in every instance where 100 or more milligrams of rutin were injected the time value after rutin was greater than before.

TABLE 1

*Cutaneous capillary permeability of trypan blue in rabbits before and after the intravenous administration of rutin*

RABBIT NO.	RUTIN  mg./kg.	TIME (MINUTES) OF APPEARANCE OF DYE WITH						
		Chloroform Wheal			Histamine Wheal		Negative Pressure	
		Before	After	Returns to Normal in	Before	After	Before	After
3	200	2	5					
6	200	4	14					
7	200	4	30					
11	200	2	4					
12	200	2	5					
4	200	2	9	90				
5	200	2	5	90				
13	200	3	14	53				
10	200	2	8	46	5	15		
8	200	6	29		7	30*		
9	200	2	23		5	19		
14	100	5	13					
15	100	1	54	60				
17	100	1	49					
18	100	6	8				3	15
19	100	3	12	51			1	30*
20	100	2	4				1	30*
21	100	3	5	27			1	2
22	100	5	8	24			1	2
16	100						1	30*
15	50	1	1					
17	50	1	2					

\* No staining of wheal at time indicated.

In the first 19 animals the average time of the dye-appearance in the chloroform-irritated areas before rutin was  $3 \pm 0.3$  minutes, and after rutin administration it was  $15.7 \pm 3.3$  minutes. Following the injection of rutin observations were made on 8 animals on the lapse of time before the dye-appearance time in newly irritated areas returned to normal. The shortest period of protection was 24 and 27 minutes in two animals, whereas two other rabbits showed some protective action on the capillaries for as long as 90 minutes.

The 3 rabbits in which histamine wheals were produced gave definite evidence that rutin delayed the onset of the staining of the wheals. Of the 6 rabbits to

which 30 mm. of negative pressure was applied for one minute 5 showed staining of the treated area immediately after removal of the suction cup, while the sixth animal showed staining in 3 minutes. After rutin administration none of the rabbits showed staining immediately following removal of the cup; two showed staining in two minutes; one in 15 minutes; and the remaining three were unstained after 30 minutes.

**Discussion.** Of the three methods employed to demonstrate the effect of rutin on capillary permeability, irritation by chloroform was the most satisfactory. In developing a method for testing permeability the use of negative pressure is objectionable since it may involve the fragility factor. The production of histamine wheals is less satisfactory than chloroform irritation because the areas involved are larger and fewer observations can be made on a given animal.

The intravenous injection of rutin in a dose of 100 mg. per kilogram of body weight gave as good protection of the capillaries as did 200 mg. Only two experiments were made using a dose of 50 mg. since the protection appeared to be much less pronounced. Under the experimental conditions employed, the demonstrable effects on capillary permeability were of relatively short duration. The effects were first noticeable about 10 minutes after injection of the rutin and lasted for a maximum period of 90 minutes. This latent period of 10 minutes suggests that the protective action involves some mechanism other than the mere presence of rutin in the tissues.

Examination of the rabbit urine, the urine of dogs which had been given rutin intravenously, and the urine of one of us (A. M. A.) after oral administration of 1 gram of rutin showed that rutin is excreted rapidly. This accounts for the short duration of the protective action.

The procedure described affords a simple method for the detection of so-called "vitamin P" properties of various flavonols and flavone derivatives when intravenous injection of the material in question is feasible. Studies are in progress on the applicability of the method when other routes of administration are required.

#### SUMMARY

Investigations have been made on the use of trypan blue for the testing of cutaneous capillary permeability in rabbits after applying chloroform to the skin; after intracutaneous injection of histamine, and after applying 30 mm. of mercury negative pressure to the skin.

The intravenous administration to rabbits of rutin in doses of 100 or 200 mgm. per kilo of body weight definitely decreased cutaneous capillary permeability as determined by the methods employed.

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# THE EXCRETION OF $N^{15}$ IN THE URINE OF DOGS AFTER THE ADMINISTRATION OF LABELED PENTOBARBITAL<sup>1</sup>

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In contrast with long-acting barbiturates such as barbital and phenobarbital which to a considerable extent are excreted unchanged in the urine, shorter-acting barbiturates such as amytal and pentobarbital (Nembutal) have not been found in the urine. Shonle and coworkers (1) were able to recover neither of the latter drugs in the urine and suggested that the probable degradation products were di-alkyl substituted acetyl urea and acetamide which "could be completely broken down into  $CO_2$ ,  $H_2O$  and  $NH_3$ ". Thus, the great advantage of using a labeled drug to determine its fate is apparent. In the case of pentobarbital it is a necessity which led to the present experiments in which pentobarbital containing one atom of  $N^{15}$  was administered to dogs and the distribution of the stable isotope in the urine was followed:

**METHODS.** For the synthesis of the labeled pentobarbital sodium, labeled urea was first prepared in excellent yield by mixing in equimolecular amounts concentrated aqueous solutions of potassium cyanate and isotopic ammonium sulfate. The resultant slurry was diluted with 8-10 volumes of 95% ethanol and shaken at  $40^\circ$  for five days. Evaporation of the supernatant alcohol gave snow-white urea, which, before use, was recrystallized from isopropanol and dried. The condensation with ethyl 1-methyl butyl malonic ester and subsequent purification were carried out as described by Volwiler and Tabern (2). The labeled pentobarbital sodium contained 14.9 atom % of excess  $N^{15}$ .

Three experiments were performed in 3 different female dogs to which 50 mgm. per kgm. of dissolved drug were administered by stomach tube. The animals lapsed into coma in about 20 minutes and began to recover consciousness about 8 hours later. Two hundred ml. of water were given by stomach tube at 0, 3, and 8 hours. The animals were fed after the 24-hour sample of urine had been collected. Before the drug was given, the bladder was emptied by catheterization. Urine was secured by catheter 3 hours and 8 hours after the drug was administered. Subsequent samples were collected after 24 hours in metabolism cages draining into bottles containing toluol. Further collections up to 48 or 72 hours were continued in 2 animals; however, the low percentage of excess  $N^{15}$  in certain fractions of these samples either was within the limits of error of estimation of  $N^{15}$  in the mass spectrometer ( $\pm 0.005\%$  of excess  $N^{15}$ ) or the accuracy of determination of  $N^{15}$  was low so that data from such samples could readily lead to an erroneous conclusion concerning the total excretion of  $N^{15}$  even after 24 hours.

The partition of the urinary  $N^{15}$  was determined as follows: Ammonia was adsorbed directly from the urine with permutit, and the ammonia, liberated by 2.5 N-sodium hydroxide in a micro-Kjeldahl distilling unit, was determined titrimetrically. Urea was then similarly determined on the washed supernatant of the same sample following the action

<sup>1</sup> This investigation was in part supported by a grant from the United States Public Health Service.

of a highly active sample of urease.<sup>2</sup> Finally, the total nitrogen was determined on an aliquot of the urine by means of a micro-Kjeldahl method. Blank determinations were always run simultaneously to correct for traces of ammonia in the permittit and in the urease. The methods were found to give satisfactory results, with excellent agreement between duplicate samples, when a standard solution of ammonium sulfate, urea, and creatinine was analyzed. Larger, but similarly prepared distillates of urinary ammonia, urea, and "total" nitrogen were digested according to the procedure of Rittenberg and Shemin prior to isotopic analysis. We are indebted to Dr. D. Rittenberg for the mass-spectrometric determinations of  $N^{15}$ .

TABLE 1

*The excretion of excess  $N^{15}$  in the urine of dogs after the oral administration of 50 mgm. per kgm. of pentobarbital sodium containing 14.9 atom % excess  $N^{15}$*

DOG NO.	DOSE OF EXCESS $N^{15}$	EXCRETION PERIOD	TOTAL URINARY EXCRETION OF					
			$NH_3$		Urea		non- $NH_3$ * non-Urea fraction	
			% total dose of $N^{15}$	mgm. N	% total dose of $N^{15}$	mgm. N	% total dose of $N^{15}$	mgm. N
	mgm.	hrs.						
1	7.89	0-3	<0.7 <sup>a</sup>	15	1 <sup>a</sup>	694	7 <sup>b</sup>	20
2	7.07	0-3	0	16	1 <sup>a</sup>	550	4 <sup>b</sup>	46
3	5.11	0-3	<0.1 <sup>f</sup>	7	0.6 <sup>c</sup>	283	10 <sup>a</sup>	72
1		4-8	0.1 <sup>c</sup>	42	1 <sup>a</sup>	900	12 <sup>a</sup>	108
2		4-8	0	23	0	538	15 <sup>a</sup>	83
3		4-8	<0.1 <sup>f</sup>	17	0.6 <sup>f</sup>	376	20 <sup>a</sup>	41
1		9-24			5 <sup>c, g</sup>	1743 <sup>g</sup>	44 <sup>a</sup>	361
2		9-24	<0.1 <sup>d</sup>	21	Tr <sup>h</sup>	1289	40 <sup>a</sup>	268
3		9-24	<0.2 <sup>c</sup>	30	0	696	27 <sup>a</sup>	49
2		25-48	<0.3 <sup>f</sup>	193	Tr <sup>h</sup>	5880	16 <sup>d</sup>	1360
3		25-48	<2 <sup>e</sup>	634	Tr <sup>h</sup>	12546	43 <sup>e</sup>	1440

\* Total N - ( $NH_3$ N + Urea N)

<sup>a</sup> Error =  $\pm$  3% of value given

<sup>b</sup> Error =  $\pm$  6% of value given

<sup>c</sup> Error =  $\pm$  25% of value given

<sup>d</sup> Error =  $\pm$  30% of value given

<sup>e</sup> Error =  $\pm$  40% of value given

<sup>f</sup> Error =  $\pm$  50% of value given

<sup>g</sup> Including ammonia.

<sup>h</sup> Significance doubtful owing to low concentration of excess  $N^{15}$ .

**RESULTS AND DISCUSSION.** The results are summarized in table 1. Within 24 hours after the oral administration of the pentobarbital sodium 59 to 71% of the total dose of  $N^{15}$  had been excreted (average 63%). The average excretion by periods was: 0-3 hours, 8%; 4-8 hours, 16% and 9-24 hours, 39%. Thus, excretion was fairly uniform and amounted to 2.5-3.0% of the total dose per

<sup>2</sup> The urease was kindly furnished by E. R. Squibb and Sons through the courtesy of Mr. R. J. Dahl.

hour in the first 24 hours. The data on the urine samples collected from animals 2 and 3 between 24 and 48 hours do not deserve the same consideration as the other samples. The total excretion of ordinary nitrogen was high—especially in dog 3—so that the error in the estimation of excess  $N^{15}$  was of the order of 25–30%.

The striking finding was the remarkably small proportion of excess  $N^{15}$  excreted as either ammonia or urea. Hence cleavage of the ring and further degradation was unequivocally demonstrated for only a small fraction of the total dose. The errors of determination of the  $N^{15}$  in urea were high. However, if the maximum values for the  $N^{15}$  excreted in this form in the first 24 hours be calculated, less than 7% of  $N^{15}$  appeared in the urea fraction, less than 1% was found as ammonia and nearly 93% occurred in the non-ammonia, non-urea fraction. This major fraction did not consist of unchanged pentobarbital. Like earlier workers, we have been unable to extract unchanged pentobarbital from dog urine following its oral administration, even though it was readily possible to recover added pentobarbital. In order more convincingly to establish that only trivial amounts were excreted unchanged, an isotope dilution experiment was performed. The pooled urine excreted by dog 1 between 4 and 24 hours was estimated to contain  $N^{15}$  equivalent to about 300 mgm. of pentobarbital. Three hundred mgm. of ordinary pentobarbital sodium were added to the urine. The pentobarbital was then extracted and repeatedly recrystallized. The excess  $N^{15}$  after the second recrystallization was 0.438 per cent, and after the fifth recrystallization was 0.442 per cent. Since the labeled pentobarbital contained 14.9 atom per cent excess  $N^{15}$ , the ratio,  $\frac{0.440}{14.9} = \frac{X}{300}$ , indicates that only 8.8 mgm. of the administered drug was excreted unchanged during these 24 hours of the first day. This amount represented only 2.7 per cent of the total of drug represented by  $N^{15}$  excreted during this period.

#### SUMMARY

After the oral administration of sodium pentobarbital (Nembutal) labeled with  $N^{15}$ , dogs excreted about 60% of the total dose of excess  $N^{15}$  in the urine during the first 24 hours. The excess  $N^{15}$  in the urinary ammonia and urea represented less than 8% of the  $N^{15}$  so that more than 92% was excreted as pentobarbital or metabolic degradation products derived from the drug. In one isotope-dilution experiment, about 3% of the total excess  $N^{15}$  in the urine was found to be pentobarbital; this finding confirms the conclusion of other investigators that only minute amounts of pentobarbital are excreted unchanged. Possible structures of the major excretion product or products remain to be considered.

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# COLORIMETRIC METHODS FOR DETERMINATION OF STREPTOMYCIN<sup>1</sup>

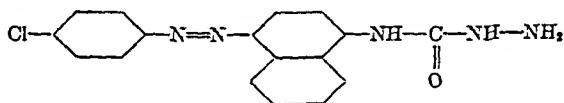
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Streptomycin may be determined by alkaline degradation to maltol and the subsequent estimation of this substance by ultraviolet spectrophotometry or by the intensity of the color developed upon the addition of ferric salts (1, 2). As this method is not applicable to solutions containing less than 4 or 5 mgm. of streptomycin per 100 ml., a more sensitive method is needed for the determination of streptomycin in body fluids.

Such a method is herein described. In principle it is based upon the interaction of the carbonyl group of streptomycin with a colored semicarbazide and subsequent colorimetric determination of the derivative so formed. The reagent used is 4-[4-(p-chlorophenylazo)-1-naphthyl] semicarbazide<sup>2</sup>.



This substance is relatively insoluble in water (less than 5 micrograms per 100 ml.), but sufficiently soluble in certain organic solvents for present purposes. When solution in methyl cellosolve (glycol monomethyl ether) is mixed with an aqueous solution of streptomycin of appropriate acidity, the semicarbazone derivative is readily formed. Following this reaction the excess of the reagent is removed by extraction with chloroform in which the streptomycin derivative is apparently insoluble. Colorimetric estimation of the colored streptomycin semicarbazone remaining in the aqueous phase is facilitated by the addition of an equal volume of concentrated hydrochloric acid which, as in the case with many other azo dyes, results in the formation of an intense blue color. The peak absorption of solutions so formed was found to be at 580 millimicrons when examined in a Beckman spectrophotometer.

The reaction between streptomycin and the semicarbazide reagent does not go to completion in homogeneous solution and a number of factors appear to con-

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Thanks are due Dr. Richard Pasternack of Chas. Pfizer and Co., Dr. Oskar Wintersteiner of the Squibb Institute for Medical Research, Dr. Henry Welch of the Food and Drug Administration, and Dr. A. C. Bratton, Jr. of Parke, Davis and Co. and for samples of salts of streptomycin and analytical and bioassay data on these. We are indebted to Dr. A. W. Goos of Cliffs Dow Chemical Co. for a generous sample of maltol.

<sup>2</sup> This is one of several hydrazine derivatives currently under investigation in this laboratory as reagents for the colorimetric or fluorimetric determination of substances having reactive carbonyl groups.



dition the extent of the reaction. As in other reactions of this type, the acidity of the reaction mixture is of considerable importance. The amount of the semicarbazide reagent necessary for the maximal formation of the semicarbazone depends on the volume of the streptomycin solution used for the determination. Thus, with the same total amount of streptomycin in 1 ml. and in 4 ml. of solution, it required about three times as much semicarbazide to form the same amount of semicarbazone in the larger volume of solution. Since, trichloroacetic acid has been found to be the most satisfactory reagent for the deproteinization of plasma prior to the determination of streptomycin by the present method, aqueous solutions of streptomycin are treated with a quantity of this reagent sufficient to yield a 3 per cent solution prior to the addition of the semicarbazide reagent. The latter contains sodium acetate and acetic acid in amounts empirically found to yield the maximum rate of conversion of streptomycin to the colored semicarbazone. With the concentrations chosen for this purpose, maximal conversion occurs in less than 15 minutes at the temperature of a boiling water bath. At room temperature the extent of this conversion in 1 hour and in 16 hours was found to be respectively but 10 per cent and 50 per cent of the maximal value.

Commercial preparations of the calcium chloride double salt of streptomycin trihydrochloride contain variable amounts of methanol and water which cannot be completely removed by conventional drying procedures without some inactivation of the base. Accordingly, the base content of a given streptomycin preparation chosen as a standard of reference must be calculated from other data. As the results of antibacterial assays are not sufficiently precise for this purpose, recourse must be had to analytical data supplied with the sample or to a determination of the maltol formed upon alkaline degradation of the base (1). This situation is further complicated by the recent discovery (3) that concentrates of streptomycin contain two components, designated A and B. As yet, information concerning the extent of occurrence of these two streptomycins in commercial preparations is lacking.

Throughout the present investigation we have utilized as a primary standard of reference an analyzed sample of the calcium chloride double salt of streptomycin trihydrochloride found to be free of streptomycin B and apparently homogeneous when assayed by Craig's (4) technique of countercurrent extraction. Determinations of the water and methoxyl content of this sample indicated that it contained 64 per cent of streptomycin (base). A quantity of this salt equivalent to 1 mgm. of streptomycin base was found to yield 0.145 mgm. of maltol upon alkaline degradation by the technique to be described later. By means of this technique and by means of the colorimetric semicarbazone procedure described below, the streptomycin content of five other preparations of streptomycin salts was determined. As will be evident on examination of the data in table 1 the values so obtained agree reasonably well with the streptomycin contents of these preparations calculated from the results of direct determinations of the water, methanol and, in three instances, the nitrogen content. Accordingly, in routine use of the colorimetric method for the determination of streptomycin described

below one may use as a standard of comparison any sample of a streptomycin salt for which adequate determinations of these quantities are available.

Under the conditions described for the determination of streptomycin by means of the semicarbazide reagent, streptobiosamine does not appear to react with the reagent. This is also true of dihydrostreptomycin which cannot so react because of the absence of a reactive carbonyl group.

Urine contains substances which interfere with the determination of streptomycin by the colorimetric method outlined above. Consequently, the determination of the streptomycin content of urine is best made by estimation of the maltol formed upon alkaline degradation of the former substance. This degradation does not occur in stoichiometric fashion, hence it is probable that the quantity of maltol formed from a given weight of streptomycin will vary as con-

TABLE 1  
*Streptomycin content of some streptomycin salts*

SAMPLE*	CALCULATED FROM			DETERMINED BY	
	Solvent Content	Nitrogen Content	Bioassay	Maltol Method	Semicarbazide Method
A	64	—	71	—	—
B	69	68	70	68	65
C	67	—	—	66	65
D	68	67	77	69	68
E	84	80	80	83	85
F	75	—	80	74	71

\* The nature and source of these six samples of streptomycin salts were as follows: A, a crystalline double salt of calcium chloride of streptomycin A, trihydrochloride mentioned in the text from the Squibb Institute of Medical Research; B, a similar salt of streptomycin from Chas. Pfizer & Co.; C, a similar salt, from Parke, Davis & Co.; D, the Food and Drug Administration Master sample of this double salt prepared by pooling preparations obtained from Merck and from Pfizer; E, an amorphous trihydrochloride of streptomycin prepared from the crystalline helianthate obtained from the Squibb Institute of Medical Research, and F, a purified sulfate prepared from recrystallized hydrochloride-calcium chloride double salt, from Chas. Pfizer & Co.

ditions selected for the conduct of this degradation are varied. The procedure developed in this laboratory for the degradation of streptomycin in both aqueous solutions and in urine allows the conversion of a constant fraction of the streptomycin present to maltol. Thus the results of assays of streptomycin salts by the maltol method and by means of the semicarbazide reagent yield results in good agreement with one another as shown in table 1.

Solutions of maltol yield a violet color upon treatment with solutions of ferric salts (4). In the presence of an excess ferric nitrate the intensity of the color formed is directly proportional to the maltol content of solutions examined. As urine contains substances which yield colored products with ferric salts, this reaction cannot be satisfactorily applied directly to urine in which maltol is formed. Interference of this nature is largely eliminated by extracting the maltol

with chloroform, in which it is readily soluble, prior to treatment with ferric nitrate solution which may be added directly to the chloroform extract. This procedure has the added advantage that it allows one to effect a considerable concentration of the maltol and thus enhance the sensitivity of the analytical method. While ferric chloride yields the same color with maltol as does ferric nitrate, it is not as satisfactory a reagent as is the latter for this purpose because of its relatively greater solubility in chloroform.

**PREPARATION OF 4-[4-(*p*-CHLOROPHENYLAZO)-1-NAPHTHYL]SEMICARBAZIDE.** The colored semicarbazide utilized as a reagent in the present method for the determination of streptomycin has not been previously described. It is prepared by the interaction of the azo dye, 4-(*p*-chlorophenyl)-1-naphthylamine, with acetone semicarbazone to yield 4-[4-(*p*-chlorophenylazo)-1-naphthyl]-1-isopropylidene semicarbazide and subsequent removal of the isopropylidene moiety by acid hydrolysis to yield 4-[4-(*p*-chlorophenylazo)-1-naphthyl] semicarbazide. The azo dye used as a starting material has been previously described (6). As it is not obtainable from commercial sources, a method for its preparation is included in the description of the synthetic methods which we have employed for the preparation of our reagent. Only the last step in the sequence of operations involved in these methods requires comment. In the final acid hydrolysis the conditions described for the conduct of this operation and the purification of the ultimate product must be carefully followed, because, incident to the hydrolysis, secondary products are formed which interfere with quantitative color formation in the final stage of the analytical procedure unless they are removed by treatment with ethanol in the fashion described.

**4-(*p*-Chlorophenyl)-1-naphthylamine.** A solution of 50 grams of sodium acetate trihydrate in 600 ml. of water was added to a solution of 14.3 grams (0.1 mole) of  $\alpha$ -naphthylamine in 400 ml. of ethanol and the mixture was cooled to 0° by means of an ice-salt bath. A solution of 12.8 grams (0.1 mole) of *p*-chloroaniline in 300 ml. of water and 30 ml. of concentrated hydrochloric acid maintained at a temperature below 5° was diazotized in conventional fashion by the addition of a solution of 8 grams of sodium nitrite in 100 ml. of water. This solution was then added dropwise to the naphthylamine solution which was continuously stirred by means of a powerful mechanical stirrer and maintained at a temperature below 5°. Stirring was continued for one-half hour after the addition of the solution of the diazonium salt. The product was separated by filtration and washed with water until the washings were free of chloride ion. This is a tedious process which requires approximately 5 hours. The dye thus obtained was dried to constant weight in an oven maintained at 70°, whereby 30 grams of material melting with decomposition at 174–181° were obtained. This crude product was recrystallized from approximately 500 ml. of boiling benzene from which it separated on cooling as fine orange needles which melted with decomposition at 187–189°. Yield, 18.5 grams (66% of the theoretical).

**4-[4-(*p*-Chlorophenylazo)-1-naphthyl]-1-isopropylidenesemicarbazide.** A mixture of 5.6 grams (0.02 mole) of recrystallized 4-(*p*-chlorophenylazo)-1-naphthylamine, 2.4 grams (0.02 mole) of acetone semicarbazone and 90 ml. of purified

xylene was refluxed for 3 hours, cooled and filtered. The solid product was washed with water and then repeatedly with ethanol until 1 ml. of the washings upon treatment with 1 drop of 6 N hydrochloric acid failed to yield a purple color. The product (5.6 grams, 74% of the theoretical) was obtained as fine orange needles which decomposed at 261–263° when placed in a melting point bath initially at a temperature of 258–260°.

Calc. for  $C_{20}H_{18}N_5OCl$ : N, 18.4; found N, 18.2

*4-[4-(p-Chlorophenylazo)-1-naphthyl]semicarbazide*. Five grams of the finely powdered isopropylidene derivative were added to a boiling mixture of 100 ml. of concentrated hydrochloric acid and 200 ml. of ethanol. The mixture was refluxed for 30 minutes, cooled and then treated with 10 grams of sodium acetate trihydrate and sufficient 6 N sodium hydroxide solution to bring the pH of the mixture to approximately 7 as estimated by Hydrion paper. After filtration, the solid product was washed with water until free of chloride ion, then repeatedly with ethanol until the washings were no longer colored red. The crude dry product was recrystallized by boiling with ethanol (1 ml. per mgm.) for 30 minutes. The alcoholic solution thus obtained was concentrated by distillation in vacuo at a bath temperature of 45–50°, until a definite separation of crystals occurred and then left overnight in an ice-box. The crystals thus obtained were recrystallized in the same manner. By this means 4-[4-(p-chlorophenylazo)-1-naphthyl] semicarbazide is obtained in variable yield approximating 25 per cent of the theoretical as an orange crystalline powder which, upon heating, darkens at 235–240° and decomposes at 260–290° depending upon the rate of heating.

Calc. for  $C_{17}H_{13}ON_5Cl$ : N, 20.6; found N, 20.6

DETERMINATION OF STREPTOMYCIN IN PLASMA *reagents*. 1. 0.133 gram of 4-[4-(p-chlorophenylazo)-1-naphthyl] semicarbazide prepared as above described is dissolved in approximately 50 ml. of redistilled methyl cellosolve by warming to 50°. 2.66 grams of sodium acetate trihydrate are dissolved in the solution so obtained, then 8.3 ml. of glacial acetic acid is added. The mixture is brought to room temperature and diluted with methyl cellosolve to 100 ml.

2. Chloroform, reagent grade

3. Concentrated hydrochloric acid

4. Trichloroacetic acid, 15 grams in sufficient water to yield 100 ml. of solution.

The procedure described below can be applied to aqueous solutions of streptomycin salts or to deproteinized plasma. It is not suitable for the determination of streptomycin content of urine.

Plasma (1 ml.), obtained from oxalated blood, is diluted with 3 ml. of water, precipitated with 1 ml. of 15 per cent trichloroacetic acid, and after standing for 20 minutes, the mixture is centrifuged. Three ml. of the plasma centrifugate so obtained or a like volume of streptomycin solution containing 3 per cent of trichloroacetic acid are added to 3 ml. of the semicarbazide reagent contained in a glass-stoppered centrifuge tube graduated at 3.5 ml. The tube is heated for 15 minutes in an actively boiling water bath, then cooled by immersion in ice-

water. Ten ml. of chloroform are added to the reaction mixture and the stoppered tube is shaken at least 100 times. After separation of the two phases the chloroform is removed with the help of a pipette whose tip has been drawn out to a fine capillary. This extraction is repeated with two more 10 ml. quantities of chloroform and sufficient water added to the aqueous phase to bring its volume to 3.5 ml. After mixing, 3 ml. of this solution are treated with 3 ml. of concentrated hydrochloric acid and allowed to come to room temperature. The maximum color develops immediately and is stable for at least 1 hour. The color intensity is determined in a Klett-Summerson colorimeter equipped with a 580 millimicron filter. In this connection, it should be noted that the refractive index of aqueous solutions of hydrochloric acid and water differ from that of water sufficiently to cause an error in the colorimeter reading when the light in the optical system is not collimated. This error is eliminated by setting the zero of the colorimeter with a mixture of equal volumes of concentrated hydrochloric

TABLE 2  
*Reproducibility of method*

NO. OF DETERMINATIONS	AMOUNT OF STREPTOMYCIN	AVERAGE SCALE READING KLETT-SUMMERSON	AVERAGE DEVIATION OF SINGLE DETERMINATION*
	<i>micrograms</i>		<i>per cent</i>
9	100	300	2.7
8	50	148	2.2
15	25	76	3.0
5	12	36	8.7
7	6	18	5.0
6	3	9	4.2

\* The average deviations of single determinations were calculated by dividing the sum of all deviations from the average by the number of measurements.

acid and a solution obtained by adding 30 ml. of methyl cellosolve to 100 ml. of water.

Data on the reproducibility of this method and the proportionality of the intensity of color formation when applied to aqueous solutions of pure streptomycin are given in table 2. Application of this method of analysis to known solutions of streptomycin in human plasma allows reasonably good recovery as shown by the data of table 3. Normal plasma yields a blank upon treatment with the semicarbazide reagent. Plasma obtained from the blood of thirteen normal humans yielded a blank, calculated as streptomycin, of 0.27 mgm. per cent with an average deviation of a single determination of 0.08 mgm. per cent.

**DETERMINATION OF STREPTOMYCIN IN URINE.** Two variations in procedure have been devised to allow analysis of urine samples containing different quantities of streptomycin.

*Reagents.* Sodium hydroxide, 2.5 N  
Hydrochloric acid, 4 N  
Chloroform, reagent grade  
Ferric nitrate, stock solution.

Dissolve 0.5 gram ferric nitrate nonahydrate in 100 ml. of 0.035 N nitric acid. For use in the first of the two procedures described below 25 ml. of this stock solution are diluted to 100 ml. with water to yield "concentrated iron reagent." For use in the second of these procedures 5 ml. of the stock solution are diluted to 100 ml. with water to yield "dilute iron reagent."

*Procedure 1.* Three ml. of diluted urine (containing no more than 2 mgm. of streptomycin) are mixed with 0.7 ml. of 2.5 N sodium hydroxide in a 125 ml. glass-stoppered pyrex bottle. This is immersed in a boiling water bath for 5 minutes, cooled in ice-water, 0.5 ml. of 4 N hydrochloric acid and 60 ml. of chloroform are added to the contents of the bottle which is then shaken for 5 minutes. Fifty ml. of the chloroform phase are withdrawn and shaken for 5 minutes with

TABLE 3  
*Recoveries from human plasma*

NO. OF DETERMINATIONS	ADDED	FOUND (AVERAGE)	RECOVERY	AVERAGE DEVIATION OF SINGLE DETERMINATION
	mgm. per 100 ml.	mgm. per 100 ml.	per cent	per cent
4	0.50	0.53	106	17
5	1.00	0.94	94	12
5	2.00	1.87	94	9
3	4.00	3.94	98	4
1	5.00	4.70	94	—
1	10.00	9.00	90	—

TABLE 4  
*Recovery of streptomycin from urine*

DILUTION	ADDED	NUMBER OF DETERMINATIONS	AVERAGE RECOVERY WITH AVERAGE DEVIATION OF SINGLE DETERMINATION
	mgm. per 100 ml.		per cent
1:5	600	4	102 $\pm$ 2.0
1:5	300	4	98 $\pm$ 2.0
1:2	150	4	96 $\pm$ 0.8
1:2	90	4	98 $\pm$ 2.8
1:2	30	4	103 $\pm$ 4.8

10 ml. of the concentrated iron reagent. A portion of the aqueous phase is withdrawn and the relative optical density determined in a colorimeter equipped with a standard green filter (545 millimicrons). The absorption peak of the iron-maltol complex is broad and reaches a maximum at 530 millimicrons. The colorimeter reading is corrected for the blank on each sample of urine used. This is determined by treating another sample of the urine in the same fashion as above described but without the addition of sodium hydroxide and heating.

*Procedure 2.* To 1 ml. of diluted urine (containing between 1 and 3 mgm. of streptomycin) contained in a 60 ml. glass-stoppered bottle 0.2 ml. of 2.5 N sodium hydroxide is added and the mixture heated in a boiling water bath for 5 minutes. After cooling by immersion in ice-water 0.15 ml. of 4 N hydrochloric

acid and 20 ml. of chloroform are added to the contents of the bottle which is then shaken for 5 minutes. Ten ml. of the chloroform phase are withdrawn and shaken for 5 minutes with 10 ml. of the dilute iron reagent. A portion of the aqueous phase is withdrawn for estimation of the intensity of color. As in procedure I, the blank value of the urine under examination is determined by extraction of a sample of the urine with chloroform, without the addition of sodium hydroxide and heating, and subsequent treatment of the extract with the dilute iron reagent.

Standardization of both of these procedures is achieved by estimation of the color intensity resulting from their application to three dilutions of a solution of a streptomycin salt of known base content. As shown by the data in table 4, these methods of analysis allow satisfactory recovery of streptomycin added to human urine.

A simplified variation of the above described procedures has proven useful for the assay of streptomycin salts. In this variation 1 ml. of an aqueous solution of streptomycin containing 1 to 2 mgm. of this substance is mixed with 1 ml. of 0.2 M sodium carbonate and heated for 10 minutes in a boiling water bath. The cooled solution is then treated with 0.4 ml. of N hydrochloric acid and diluted to a volume of 10 ml. with dilute iron reagent. The color developed is compared with that yielded by a solution of pure maltol when treated similarly but with elimination of the heating period. When assayed in this fashion, our reference sample of the calcium chloride double salt of streptomycin A trihydrochloride was found to yield 0.145 mgm. of maltol per mgm. of base. This is equivalent to 66 per cent of the theoretical yield if one assumes one molecule of streptomycin to yield one molecule of maltol. As this value is readily reproducible it offers a means of assaying the streptomycin content of salts or solutions for which other analytical data are not available.

#### SUMMARY

A chemical method for determining streptomycin in plasma and aqueous solution is described. It is based on the reaction of streptomycin with a colored semiearbazide.

Procedures for determining streptomycin in urine and aqueous solutions are described based on its degradation to maltol.

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